

Too many eggs in the Asia-Pacific basket

The decision by Australia not to fund the European Southern Observatory is a sign that its politicians see its future in science as lying in the Asia-Pacific region. That conclusion is premature.

LAST week's long-awaited "innovation statement" from the Australian Prime Minister, Paul Keating (see page 653), had one glaring and embarrassing omission: an expected contribution of A\$28 million to the European Southern Observatory in Chile. It is to be hoped that the sudden decision to drop funding for the observatory was not taken lightly, given that it was strongly backed by the Australian scientific community, including the Academy of Sciences.

Senator Peter Cook, the minister of industry, science and technology, is no doubt embarrassed by that knowledge, and probably not a little piqued at having had the presentational initiative for what is really his innovation statement snatched away by the prime minister. And only a week before the statement was made, Cook told the audience at a *Nature*-sponsored conference in Canberra that "science enhances our international prestige and image". One way to enhance his country's image is surely to make a significant (and long anticipated) contribution to the European observatory, including state-of-the-art Australian telescope technology and Australian astronomical brain-power.

One reason for Keating's decision is probably mundane. He wants to get re-elected, and he no doubt thinks he has a better chance if Australia funds several smaller research facilities in every major city than by putting significant funds into a European facility in Chile. That is not to say that the seven facilities chosen are not worthy of funding. At least one of them, the Proteome Analysis Centre in Sydney, is scientifically daring and may achieve a significant presence on the world stage. But the decision to drop support for the observatory is contrary to the scientific advice he has been given.

A more interesting and debatable implication of Keating's decision is that he and his cabinet see Australia's future collaborations in science as lying primarily in the Asia-Pacific region rather than in Europe. Keating's innovation statement was peppered with references to the Asia-Pacific Economic Forum (APEC), which has recently held its first two big meetings on science and technology in Beijing and Taipei. And the only major research facility announced last week to involve use of facilities overseas is the Synchrotron Research Programme, which has a beamline at the Photon Factory in Japan, as well as the Advanced Photon Source at the Argonne National Laboratory in the United States (also a member of APEC).

Certainly there seems to be plenty of money in Australia for scientists and science policy-makers to shuttle back and forth across the Pacific to Asia. But how substantial are Australia's links in science with its Asian neighbours and how substantial are they likely to become? The beamline at the photon factory in Tsukuba Science City is tucked away at position 31 in the corner of Japan's synchrotron facility, and has yet to make its mark on world science. And there are certainly not large numbers of Australian scientists taking up long-term residence in Japan — nor are there likely to be. The language barrier is significant and the amount of support (both money and manpower) for basic research in Japan's universities is still miserly, despite recent efforts by Japanese politicians to legislate more support for basic research.

There are state-of-the-art third-generation synchrotrons in South Korea and Taiwan that sit half empty for lack of users. But

Australians are not rushing to use them, despite the fact that Australia has already sent a delegation to the South Korean facility.

What of China? There are pockets of activity there in basic research that have sprung up in the past decade of a competitive market economy, and China may well surpass its neighbour Japan in some areas of science within a few years because of its government's more inventive management of science. But again it seems unlikely that the environment will be attractive enough to lead to substantive links in science with Australia.

The bottom line is that although the economies of Asia appear to be booming, the centre of gravity of basic research still rests very much in the West between the United States and Europe. Many Australian scientists have their roots there and it is defying the realities to think that attention can be swung to the Asia-Pacific region overnight.

At the *Nature*-sponsored conference in Canberra, Eugene Wong of the Hong Kong University of Science and Technology, a former associate director of the White House Office of Science and Technology Policy in the United States, argued that those Western economies, such as the United States, that have invested heavily in basic research obtain the best returns on more general public investment in terms of growth of the economy. Asia-Pacific economies may be booming, but sustaining such growth requires huge and inefficient reinvestment because of their lack of a science base, says Wong.

Keating may have won a few votes with his statement last week. But it reveals an inadequate appreciation of where, realistically, the best international interests of Australia's science still lie. □

Postscript from a new hand

Although *Nature* is about to enter a period of change, its values are not.

IN last week's issue, my predecessor ("an old hand") signed off. In his valedictory leading article, John Maddox thanked "a host of readers and contributors for correspondence that has always been illuminating and often entertaining". It would be inappropriate for me to attempt an overall assessment of his formidable achievements as Editor of *Nature*, but illumination and entertainment have undoubtedly been among them. Nor do I intend to outline specific plans for the future — changes are under way, but new developments are often best left to announce themselves.

Changes in style are certain, however, as is the appearance of new kinds of content. Resources are being invested in enhancing further the readability of what we produce. And there are core strengths that readers can continue to be sure of: the calibre of our authorship in "the front half", for example, and the very considerable talent and man-hours applied so as to combine speedy turnaround with properly considered decision making across all the disciplines published in "the back half". Above all, *Nature*, an entity much more significant than any individual Editor, will continue to pursue scientific excellence and journalistic impact with vigorous independence.

Philip Campbell



NASA panel draws up 'road map' to hunt Earth-like planets in space

Washington. An ambitious plan to search for Earth-like planets around other stars — and perhaps detect life on these, if it exists — is gaining momentum within the US National Aeronautics and Space Administration (NASA), and could become a central part of NASA's science programme for the next decade.

The proposed search strategy, which would begin with ground-based observations and lead early in the next century to large, space-based interferometry arrays, has still to be presented to Daniel Goldin, the administrator of NASA. But Goldin is said to be enthusiastic about the idea in principle, and is expected to unveil details of the plan at an American Astronomical Society meeting in San Antonio, Texas, next month.

A team headed by Charles Elachi of NASA's Jet Propulsion Laboratory (JPL) has spent much of this year drawing up a 'road map' for the search programme, known as Exploration of Neighboring Planetary Systems (ExNPS). Three independent teams, led by Roger Angel of the University of Arizona, Robert Reasenberg of the Smithsonian Astrophysical Observatory and Charles Beichman of JPL, were asked to come up with their own plans for a search.

These sets of proposals were then merged into a single strategy by Elachi's team. This strategy was in turn reviewed by an outside panel headed by Charles Townes of the University of California at Berkeley, who will present his group's assessment of the Elachi report — expected to be

favourable — to Goldin next week.

The ExNPS study outlines a phased approach to a planetary search, beginning with the indirect detection of large planets between the sizes of Uranus and Jupiter using ground-based astronomy and radial velocity measurements. Large telescopes with advanced adaptive optics may be able to capture images of Jupiter-sized objects directly, according to the study.

Space-based astrometric interferometers, as well as the use of gravitational "microlenses" to magnify distant star systems, would be able to detect even smaller planets, down to the size of Earth. The final phase of the programme would place a large infrared interferometer in deep space (perhaps as far as Jupiter's orbit) to search for the spectral signature of water, ozone, and other signs of life around Earth-sized planets. The ExNPS search would concentrate on the nearest 1,000 stars, out to a distance of approximately 42 light years.

None of these steps, according to the ExNPS team, requires great leaps in technology, although they do pose engineering challenges. A key guideline given to the Elachi team by NASA headquarters was that the programme be "visionary, but fiscally realistic", according to a draft of the team's report.

How NASA might structure and pay for such a programme is still to be determined. But the agency is already reorganizing its science office, with 'origins' as one of the key themes. Edward Weiler, at present

programme scientist for the Hubble Space Telescope, will head the new directorate, which will address a wide range of questions, from galactic evolution to the origins of life.

According to Weiler, ExNPS is only one proposed direction for the office. Another NASA task force has called for the development of an astrometric interferometer in Earth orbit. And a study led by Alan Dressler of the Carnegie Institution for the Association of Universities for Research in Astronomy (AURA) will soon recommend that the agency pursues a next-generation space telescope at least four metres in diameter, which could reveal information on the first billion years of galactic formation. NASA's Goddard Space Flight Center in Maryland has already begun a year-long study to see if such a large space telescope could be built for as little as \$500 million.

Weiler sees these programmes as complementary, with the astrometric interferometer, for example, fitting snugly into the ExNPS roadmap. But a large space telescope, even a relatively cheap one, could compete with a planetary search for a place in the funding queue.

Weiler emphasizes that "we would like to do this programme without having to look for new money" and that ExNPS "won't be an issue for a Congressional new start for at least one or two years". The earliest the astrometric interferometer could start development, he says, would be 2000.

Meanwhile, the ground-based search could begin at the Keck Observatory in Hawaii (funded partly by NASA) and at other large observatories now in the planning stages. The Space Infrared Telescope Facility (SIRTF), scheduled for launch in 2002, also contributes to the ExNPS programme, says Weiler, as it tests cryogenic technologies needed for a space-based interferometer and provides critical data on the dust surrounding candidate stars.

Weiler says that it is too early to estimate the overall cost of ExNPS. But he believes it could be carried out for between 10 and 20 per cent of NASA's space science budget over the next 20 years. That number could be lower still if international partners are brought in, according to members of the ExNPS team. The European Space Agency has a similar concept for a space infrared interferometer, called Darwin, under study.

But aside from funding concerns, the ExNPS report is expected to come to be seen as a milestone in NASA's history, namely as the space agency's official statement that the detection of Earth-like planets is now both scientifically and technically feasible.

Tony Reichhardt

Australian institute gets new director

London. **Suzanne Cory, a molecular geneticist, will become director of the Walter and Eliza Hall Institute of Medical Research in Melbourne, Australia, it was announced this week. Cory (right) will succeed Sir Gustav Nossal, the current director, when he retires in June 1996.**

Cory, who is currently professor of molecular oncology at the institute, received her PhD at the University of Cambridge for studies at the Medical Research Council's Laboratory of Molecular Biology. She will be the fifth director of the Hall institute, Australia's foremost biomedical research institute.

Having returned to Australia in

1971 with Jerry Adams jointly to found the Molecular Biology Unit at the Hall Institute, Cory has established an international reputation for her contributions to the molecular genetics of antibody production and of leukaemia development.



Cory expects the Hall Institute to continue working on its current main areas of research, such as blood cell development and leukaemogenesis, basic immunological mechanisms and their derangement in autoimmune disorders, and host-parasite interactions. She also says the institute is likely to pay greater attention to the molecular genetics of disease susceptibility. □

Boost for French universities following student protests

Paris. France's embattled government last week conceded to the main demands of striking university students and staff by agreeing to provide the universities with a FF4-billion (US\$800-million) rescue package and organize a wide national consultation process aimed at producing a government-endorsed white paper (policy document) on the future of French universities.

The new package — the fourth to be proposed in under six weeks by François Bayrou, the minister for higher education — offers 4,000 new staff posts next year, FF369 million more to pay for running costs in 1996, and an immediate FF2 billion for renovations needed to help bring university buildings up to minimum safety standards. Bayrou's first proposal, an emergency plan to help the worst-off universities, offered just 400 new posts and FF160 million.

The climb-down seems to have been prompted by the prospect that protests by the increasingly organized student movement might add momentum to the massive public sector strikes that have paralysed transport and other services throughout France over the past few weeks. The spectre of the student 'revolution' of May 1968 still haunts French politicians.

Bayrou's latest offer follows two months of growing student unrest. This began on 9 November with a strike by students and staff at the University of Rouen, and has since spread to more than 20 universities, leading to the largest student demonstrations in over a decade. The usually discreet Conference of University Vice-Chancellors (CPU) has also been outspoken in its support of demands for more staff and buildings.

The CPU has welcomed Bayrou's proposals — which meet most of its demands — with "great satisfaction", describing them as a "strong sign of a recommitment by the state" to the university system. Similarly, the minister's proposal for a wide national consultation process aimed at producing a white paper on the universities, to include a commitment to multiannual funding, has been cautiously welcomed by many observers as an indication that the government may be ready to make higher education a priority.

The dispute stems chiefly from the failure of government investment in the universities to meet demand in recent years. France has spent more than FF32 billion over the past five years in building more than 1.5 million square metres of new university space under the *Université 2000* scheme, an ambitious plan to reconstruct the university system.

But even this huge investment has fallen far short of needs. Student numbers increased by a quarter during the same period, while teaching staff grew only by 15 per cent.

University buildings are also in dire decay, one in ten falling below minimum

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REASONS

Revolting: students added to pressure for funding to increase staff posts and renovate buildings.

safety standards, and classrooms overcrowded, according to a report commissioned last year by François Fillon, the then minister for higher education. The report said that some buildings should be closed, which would further increase overcrowding. Adding to the nightmare of accountants at the education ministry, a study this month estimated the cost of removing asbestos from the 200,000-square-metre campus at Jussieu in central Paris at FF880 million.

On the bright side, pressure on universities is likely to ease as student numbers decrease in line with the fall in the number of births that started around 1974. Similarly, staff retirements should open up many new junior teaching posts.

But Bayrou's fourth plan still falls short of meeting the demands of the *coordination nationale*, a body created by student groups during the dispute to provide them with a single voice. It has demanded, for example, that the government create 8,000 new posts next year, free FF4 billion for immediate renovation work, and commit itself to a plan to spend FF50 billion over five years.

The *coordination nationale* has rejected the latest proposals as "insufficient", and called for students to both continue the dispute and widen it to include support for the public sector demonstrations scheduled for this week. But the movement has been weakened by in-fighting, leading last week to the breakaway of UNEF-ID, the main moderate left-wing students' union.

Events this week show whether Bayrou's proposals will take the steam out of the students' protests. There is some evidence, such as the end of strikes at several universities, that this may happen. **Declan Butler**

India plans to recoup investment through satellite services

New Delhi. The Indian Space Research Organization (ISRO) says that earnings generated over the next seven years by its latest satellite, INSAT-2C, launched successfully last week by Europe's Ariane rocket, are expected to surpass the total investment India has so far made in space.

There is no reason to doubt this optimism. The single objective of INSAT-2C is to make money by supporting the information and telecommunications industries. In contrast, two earlier satellites in the INSAT-2 series had other aims in addition to providing telecommunication services. For example, both carried meteorological payloads intended to take photographs of clouds and thus provide early warning of imminent cyclones.

This payload was dropped from INSAT-2C. Instead, the 2.1-tonne spacecraft was packed with 24 transponders in order to achieve maximum returns from each kilogram of payload placed in orbit. Leasing one transponder alone will generate US\$2 million a year. ISRO has already leased 10 such transponders of the planned INSAT-2E to the international consortium INTELSAT for US\$100 million. This satellite is due to be launched in 1997.

The main focus of INSAT-2C is to generate revenue by providing new services to India's business community. Three of the transponders operating in the Ku band (11–14GHz) were introduced for the first time in INSAT-2C to provide direct links between city exchanges and data services using very small antennas no more than 50 cm in diameter. The satellite will also meet the communication needs of railways, trucking services and the shipping industry, as well as individuals on the move.

INSAT-2C cost US\$40 million to build and a further US\$75 million to launch. It will also be a major asset for the state-controlled television service, Doordarshan. According to ISRO, the satellite will allow Indian programmes to be transmitted for the first time to an area extending from north-east Africa to south-east Asia. This broadened audience is expected to generate a substantial income from advertising.

With 12 of the 24 transponders allocated for Doordarshan's television service, ISRO officials say that INSAT-2C will be a major competitor to the recently launched ASIAT-2, on which Rupert Murdoch's News Corporation International has already leased eight transponders. Doordarshan has been fighting to regain its share of the audience and revenue lost to Murdoch's STAR TV network. It expects to use five channels for its programmes, and to lease out the rest. **K. S. Jayaraman**

Germany seeks fusion plan compromise ...

Munich. The German ministry of research may be prepared to compromise on efforts to place a strict cap on funding for national fusion research. If so, the move may help to break a deadlock in year-long negotiations between the ministry and the Max Planck Institute for Plasma Physics (IPP) in Garching, over the funding of the IPP's new fusion reactor, the Wendelstein-7X, which is to be built in east Germany.

The Wendelstein-7X is the 'next generation' stellarator, one of several fusion reactor designs under test around the world, which will replace IPP's current stellarator, Wendelstein-7AS, in Garching. Its DM400-million (US\$275-million) investment costs are to be shared by Germany and the European Commission as part of the commission's fusion programme.

Construction of the reactor was approved by the programme's scientific committee last year, and detailed costings were approved by the commission in October. As part of the German government's post-reunification policy of siting all major new technological projects in east Germany, the reactor will be sited at Greifswald, in the north-eastern state of Mecklenburg-Vorpommern.

Mecklenburg-Vorpommern has agreed to pay the additional DM120 million required to provide an infrastructure on the 'green-field' site at Greifswald comparable to that which already exists at Garching. Half of the planned staff of 300 would move from Garching over the next few years.

But despite such previous agreements, the federal government has been dragging its feet. Jürgen Rüttgers, Germany's research minister, who took office in November last year, says that he inherited an agreement whose costs he considered to be excessive. He asked for the costs to be reassessed, even though fusion researchers argued that it was too late to change the technical specifications.

Tension became public in the autumn, when the federal government refused to pay out the first building costs of DM12 million for next year, to save money. The project was bailed out in October by Mecklenburg-Vorpommern, which plans to reclaim the money later from the government.

Tensions rose still higher last month, when Rüttgers was quoted in a newspaper interview as saying that he did not ask technical people how much money they wanted, but rather told them how much money the government was prepared to give them. He added: "Now they are saying that the money

is insufficient, and I simply don't understand this position."

The statement, coming during prolonged and tense negotiations over the stellarator project, caused consternation among fusion scientists. Klaus Pinkau, director of the IPP, says that the minimum costs had been checked and approved by the commission, and that "there were no hidden luxury costs like golden bath taps" to be negotiated.

Pinkau says that the basic issue is whether the rate at which the money is spent can be spread out so that it does not exceed the maximum spending target of DM200 million per year for fusion research that was set by the federal government in 1986. But, like other fusion researchers, he

is concerned that this target was never linked to inflation. Such a correction would have increased the spending target to DM270 million by 1996, he says.

But there are now signs that the government may be prepared to soften its original stand that the ceiling must not be exceeded. According to one ministry official, the government is prepared to accept that its total spending on fusion research will have to increase as from 1997, and has also agreed that the pace of the programme should not be reduced to spread out the costs. As a result, he says, and assuming no technical difficulties, the original goal of having the Wendelstein-7X operational in 2005 should be achieved.

Alison Abbott

...as US faces up to doubts over ITER

Washington. The US Department of Energy (DoE) is considering withdrawing from participation in the International Thermonuclear Experimental Reactor (ITER), a project widely considered as crucial for the future of fusion energy, following a one-third cut in its budget for fusion research.

Martha Krebs, the DoE's director of energy research, told her Fusion Energy Advisory Committee last week that, with little prospect for full-scale US participation in ITER's construction phase, continued US participation in the current engineering design phase should be questioned as well.

With the DoE's fusion budget expected to remain between \$200 million and \$250 million for several years, she said after the meeting that it was "highly unlikely" that the United States would participate in the construction of ITER. "Then you might say, why should we do the EDA [energy design analysis], or how can we do it in a way that could support our base programme?"

But Krebs admitted abandoning ITER would cause concern in the White House, whose science advisers are still keen that the United States should be seen as a "reliable partner" in international science projects.

The United States currently contributes \$70 million a year to the ITER engineering design activity, which is being carried out jointly with Europe, Japan and Russia, and is scheduled to conclude in 1998. No decision has yet been taken on whether to build the test reactor, which is estimated to cost around \$10 billion.

Faced with becoming a second-rate fusion power, the United States may have to find a 'niche' role in global fusion efforts, warned Anne Davies, associate director for fusion energy in the DoE's Office of Energy Research. The European Union will spend about two-and-a-half times more than the DoE's \$244 million on fusion research this year, and Japan will spend roughly 50 per

cent more than the United States. Russia spends less, but virtually all of its programme is focused on ITER.

Despite the importance of ITER to the future of fusion, Davies insists that she "would never recommend" devoting the entire US fusion budget to ITER construction. "I could see us carrying a \$50 million [component] into ITER construction," she added. "[But] our partners would have to decide whether it is worth having us as a partner for such a limited contribution."

Davies also revealed that her office had drafted a plan for an experimental reactor that would cost roughly half ITER's estimated \$10 billion. But she said that her counterparts from the other ITER participants had given her the cold shoulder when she had approached them informally with the idea. They showed no interest in discussing the "downscoped" project, she said, calling the plan a "non-starter".

According to Davies, the study, which cost around \$700,000, concluded that a tokamak reactor with conventional copper coil magnets could be built for around \$4 billion. But this would only be capable of performing half of ITER's experimental role, namely studying the physics of ignited plasmas. The other function to be carried out by ITER — the development of materials and technologies required for a working fusion power reactor — would not be addressed by the less costly device.

The DoE study resulted from a recommendation last summer by a panel of the President's Council of Advisers on Science and Technology (PCAST) that ITER be scaled down to halve its costs, thereby allowing full US participation within constrained budgets (see *Nature* 377, 567; 1995). The recommendation has stunned and upset the other ITER parties, whom Davies acknowledges remain committed to proceeding with the full-scale experiment.

Dave Kramer



Rüttgers: wants check on stellarator costs.

Australia backs innovation, shuns telescope

Sydney. In a move designed to stimulate both science and technological innovation in Australia, the prime minister, Paul Keating, last week announced the launch of several new national and international science facilities and projects.

These include Australia's first dedicated centre for genome research, and another for mass screening of proteins. But Australian and European astronomers were disappointed that an expected contribution to the European Southern Observatory's Very Large Telescope (VLT), currently under construction in Chile, did not materialize.

Keating made what he called an "innovation statement" at a business conference in Melbourne. Its main contents, however, were completed several months ago, and their presentation had been timed to give maximum benefit to the ruling Labor Party's general election campaign early next year.

In the intervening period, several significant changes were made. Last year, for example, the government called for proposals for several 'major national research facilities'. More than 60 applications were received, including some projects involving facilities overseas, and these were reduced to 16 about six months ago.

At that time, it was widely anticipated that a proposal to contribute A\$28 million (US\$20.35 million) to the VLT, to include the construction of low-noise receivers built in Australia, would be selected from the short-list.

But the telescope proposal has been dropped for several reasons. One is that it would have consumed almost half of the A\$60 million set aside for the 'national' facilities, and would have severely limited the number of others that could be funded.

The more important considerations, however, appear to have been political. Australia's politicians are keen to develop economic, trade and science links with countries in the Asia-Pacific region, following a successful meeting of the Asia-Pacific Economic Forum (APEC) in Osaka, Japan, last month.

Both the Australian cabinet and the prime minister are said to have considered the word 'European' to be unattractive in this political climate. With a general election looming, many Australian scientists suspect they were also keen to maximize the overall number of national facilities, so that one major facility could be given to every major city in Australia, namely Sydney, Canberra, Melbourne, Brisbane, Perth, Hobart and Adelaide.

The seven facilities chosen, with a total funding of A\$62.4 million, include a new genome research facility with 'nodes' at the University of Queensland in Brisbane and

the Walter and Eliza Hall Institute of Medical Research and Royal Melbourne Hospital in Melbourne. This facility will carry out major gene sequencing and mapping projects, and will received Aus\$10 million.

A further A\$7 million will be spent on the 'Proteome' analysis centre at Macquarie University in Sydney for rapid mass-screening of proteins (see below).

A sum of A\$12.2 million is to be spent on a synchrotron research programme, which will provide Australian scientists with access to an upgraded beamline at Japan's Photon Factory in Tsukuba Science City, and a new beamline at the Advanced Photon Source at Argonne National Laboratory in the United States. This also includes funds for a feasibility study of an Australian synchrotron.

Other facilities include:

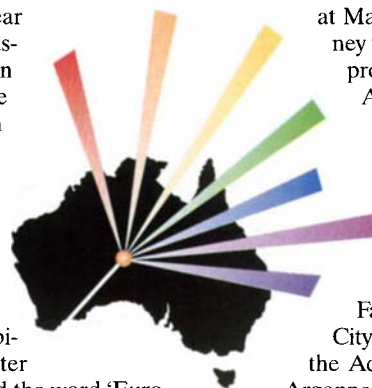
- the Airborne Research Australia Facility, at South Australia's Flinders University, which will carry out atmospheric and meteorological research, including studies of the ozone layer over Australia;
- a Plasma Fusion Research Facility, an upgrade of the Helix Plasma Facility at the Australian National University (ANU) in Canberra, where the 'stellarator' approach to fusion is being developed; and
- a National Seismic Imaging Resource for generating three-dimensional images of the Earth to depths of more than 1,000 km, which will have facilities and equipment based at the Australian Geodynamics Cooperative Research Centre in Perth, the ANU's Research School of Earth Sciences and the Australian Geological Survey.

As something of a consolation prize for astronomers, the Australia Telescope will get A\$11 million for an upgrade — A\$1.3 million more than it had requested. These funds will be used to extend the telescope's wavelength range and angular resolution by a factor of ten, and will involve all the telescope's sites in New South Wales.

The extra money will also be used to convert the Telstra antenna in Ceduna, South Australia, and the University of Tasmania's radio telescope at Hobart to create a six-station Australian network of antennae for very-long-baseline studies.

Astronomers hope that they may be able to salvage a few million dollars from the larger-than-expected budget for the upgrade so they can contribute something to the European Southern Observatory in Chile. But this would still be an order of magnitude less than originally hoped for.

David Swinbanks



Government backs proteome proposal

Sydney. A year ago, a proposal by Keith Williams of Macquarie University in Sydney, New South Wales, was little more than a dream. But good fortune — and the ill-fortune of astronomers — has given him time to demonstrate that his idea of mass screening and sequencing proteins, much like the rapid sequencing of DNA, is both feasible and fast enough to provide an alternative approach to the molecular analysis of whole organisms.

The term 'proteome' was coined by Marc Wilkins, a former PhD student of Keith Williams, to describe an entire organism's protein complement. Williams's idea is to screen and determine all the proteins produced by the DNA of an organism, rather than using the more conventional approach of sequencing DNA in order to determine the genes that produce proteins.

The protein approach provides important additional information, as, after their translation from DNA, many proteins are modified by sugars and phosphates. Such changes can have

profound effects on the biological function of proteins.

Until very recently, the technology required for rapid analysis of proteins lagged far behind that used for DNA analysis. But working with companies such as Beckman Australia and GBC Scientific Instruments (formerly part of ICI Australia), Williams's laboratory has developed many new machines for the rapid analysis of proteins, using a hierarchical approach progressing from the highest throughput and cheapest technology to the most expensive and time-consuming, depending on how much information already exists about a given protein.

In addition, his laboratory can handle milligram quantities of samples on two-dimensional electrophoresis gels that yield individual spots of pure protein of sufficient quantity for analysis. He claims to be able already to screen a hundred proteins a week, and that the new facility will screen a hundred a day at A\$2 a protein.

D. S.

Fast reactor leak hits nuclear safety image

Tokyo. A fault in the cooling system of Japan's prototype fast breeder reactor Monju forced its shutdown last Friday, 8 December, causing concern about the safety and reliability of the technology and prompting calls for a review of Japan's nuclear power policy. A fault in the secondary cooling system allowed between two and three tonnes of liquid sodium to leak out, causing a temperature abnormality that triggered a fire alarm.

According to officials of the semi-public Power Reactor and Nuclear Fuel Development Corporation, the plant, located in Fukui Prefecture on the Japan Sea, was shut manually on Friday evening after an alarm had gone off. A task force headed by the plant's chief manager was immediately set up, and workers were ordered to drain about 80 tonnes of liquid sodium into a tank in order to prevent further leakage. Oxidized sodium was subsequently found in piles beneath the liquid sodium pipes.

The source of the leakage has yet to be

established because of the insulation and panelling covering the pipes. But officials have suggested that the most likely site may be a weld connecting the system to a branch pipe equipped with a device that measures the temperature of the sodium.

All of the piping in the section had to be replaced in 1992 due to faulty construction. This, together with the fact that the plant just began operating, has led to speculation that the cause was faulty construction, rather than metal fatigue or another cause.

Monju, which was operating at 40 per cent of its 28-MW capacity at the time of the accident, started operating in August, and according to current plans will reach full capacity in June 1996. Construction of the ¥600-billion plant began in 1985. The Japanese government is hoping to follow Monju with the introduction of commercial reactors around the year 2030. This fast breeder reactor programme is a central plank of its energy policy, but has met opposition both domestically and internationally.

The accident has provoked strong reactions from local and prefectural governments, as well as local citizens' groups, which have demanded that the operating authorities should publish all information related to the plant and its operations, and participate in regular discussions with concerned parties. Technical problems related to the liquid sodium cooling systems have plagued fast breeder reactor programmes in France, Germany and the United Kingdom, all of which have either cancelled or severely reduced their fast breeder programmes.

Apart from concern about safety and the environment, the programme has also met resistance from the Japanese power utilities, which resent the high costs of the programme, part of which they must bear. Internationally, the programme has come under fire because of the huge amounts of plutonium — which could be used for nuclear weapons — that Japan will accumulate as the programme develops.

Stephen Barker

Researchers split over food safety as schools ban beef

London. The British government has again been forced to deny claims that bovine spongiform encephalopathy (BSE) — widely referred to as 'mad cow' disease — can be transmitted to humans, following a decision by local education authorities representing more than a thousand schools to stop serving beef.

The move follows public statements by prominent scientists and health experts who have indicated that, although no evidence of such transmission has been found in laboratory tests, the risk — that BSE might be associated with its human equivalent, Creutzfeldt-Jakob Disease (CJD) — is sufficient for them to stop eating beef.

Addressing the House of Commons last week, the prime minister, John Major, repeated the government's position that "there is currently no scientific evidence that BSE can be transmitted to humans or that eating beef causes CJD in humans". He added: "I am also advised that beef is a safe and wholesome product."

Kenneth Calman, the government's chief medical officer, acknowledged that the annual number of CJD cases had doubled to 55 in the past decade in the United Kingdom. But he pointed out that this is still within worldwide averages, and almost certainly due to better detection and not through eating products infected with BSE.

The government's view is shared by many scientists. One CJD researcher, for example, expressed surprise that schools had chosen to ban beef "one decade after BSE had first been reported" and three years after the incidence of BSE had peaked. He pointed

out that new cases of BSE have now dropped from 1,000 a week to 300.

But such views have had little impact on the public. The chairman of one local authority education committee in the north of England, for example, said his authority has a legal requirement to ensure that children are given food that was considered safe to eat. The authority, he said, will not serve beef until "the government can prove with concrete research results that people cannot get CJD after eating BSE-infected food".

The current scare follows the recent deaths of four farmers and two schoolchildren from CJD. The government's apparent lack of concern has, to some, been underlined by its decision to cut the budget of a

laboratory conducting research into CJD, the Edinburgh Neuropathogenesis Unit, part of the Institute of Animal Health, with the possible loss of 15 research staff. The Ministry of Agriculture, however, has promised to increase the total BSE/CJD research budget by £1 million to £6.4 million next year.

The BSE infecting agent is a 'prion', an infectious protein that attacks the brain, leaving it sponge-like. It is found in brain and spinal cord. Since 1989, the use of offal in human food and in cattle feed has been banned, but not completely eliminated.

Last week, several scientists, including Sir Bernard Tomlinson, a neuropathologist and former government medical adviser, said they had changed their minds about the possibility of a BSE/CJD link and had stopped eating food containing beef offal.

In a full-page newspaper article, Colin Blakemore, Waynflete professor of physiology at the University of Oxford, said the government was wrong to transform "cautious scientific and medical advice into categorical reassurance". Not only is there a conceivable risk of BSE being transmitted from cows to humans, he said, "but it is increasingly being acknowledged by experts". Blakemore advised the public to stay calm but "consider giving up all beef until the picture is clearer".

But James Ironside, a neuropathologist with the CJD Surveillance Unit at the Edinburgh Western General Hospital, says such statements carry authority and "could be potentially misinterpreted by the public".

Ehsan Masood



Richard Greenhill

Off the menu? Beef is safe, say government advisers, despite fears surrounding BSE.

Hyping results 'could damage' gene therapy

Washington. US biomedical researchers and their sponsors have been criticized for "overselling" the result of somatic gene therapy trials by a leading advisory panel to the National Institutes of Health (NIH), which has called for greater emphasis to be given to more basic, rather than clinical, research in the field.

The conclusions have come as little surprise to researchers involved in this work, partly because of their earlier release in draft form, and partly because they coincide with the views of Harold Varmus, the director of NIH, who set up the *ad hoc* panel to assess the NIH's investment in this rapidly expanding field.

But the panel's views are still likely to have considerable influence on future patterns of NIH research funding. Varmus will soon set up a new panel, provisionally called the NIH Gene Therapy Coordinating Group and including the directors of several NIH institutes, to study how to implement the panel's recommendations.

Up to 4 December, the NIH's Recombinant DNA Advisory Committee had approved 136 clinical protocols involving gene transfer, while 910 subjects had undergone NIH-supported gene transfer trials in the United States, and 1,024 had undergone trials worldwide. The report, presented to a meeting of the NIH director's advisory committee last week, acknowledges that gene therapy offers "extraordinary" long-term potential for managing and curing many diseases.

At the same time, however, the panel warns that a rush of prematurely optimistic publicity risks eroding public confidence and damaging the field. Investigators and their supporters, it concludes, should therefore be

"more restrained" in public discussion of their findings and the prospects for gene-based therapies. "There was a uniform feeling that there has been an overselling of current research in the field, which has led to an inaccurate perception of success," says Stuart Orkin of Harvard Medical School, who co-chaired the panel.

According to the panel, excessively optimistic public perceptions may in turn be causing patients to make poor therapeutic and reproductive decisions, in the belief that cures are imminent. Indeed, it says that, despite anecdotal claims to the contrary, clinical efficacy has not been definitively demonstrated in any gene therapy protocol.

Reflecting this conclusion, the report calls for increased emphasis on basic research. "In the enthusiasm to begin human gene therapy trials soon after gene discovery, important aspects of disease pathophysiology, cell biology and biochemistry have often been under-emphasized," it says.

In particular, the panel calls for more research on gene transfer and expression. It recommends "vigorous" and expanded research to improve vectors for gene delivery. The panel urges greater focus on maintaining high-level expression of transferred genes, directing gene transfer to specific cell types, and an understanding of how recipient cells handle and express foreign DNA.

The report also urges strict adherence to high standards in clinical protocols. "Inherent in that [proposal] is the suggestion that we don't think that's been the case so far," says Orkin. "Some [clinical studies] need to be done. But they must be informative and of high quality," he said after the meeting.

The committee complains that the poor design of clinical studies often means that

they do not yield any useful information when they fail — for example, when no detectable gene transfer occurs. But it nevertheless stresses that there exists a "clear and legitimate" need for clinical studies, partly because trials with animals cannot always be extrapolated to humans.

The 14-member panel, which had been asked to carry out a broad review of gene therapy research and develop recommendations on NIH-sponsored research, spent seven months preparing its report. Varmus



described last week it as "exceptionally good," and said that he agreed with its warning about publicity. Without evidence of the efficacy of gene therapy, he said, "an extrapolation from hope to hype is really not appropriate".

But the conclusions have already come under fire from some practising clinicians. Malcolm Brenner, for example, director of the cell and gene therapy programme at St Jude's Children's Research Hospital in Memphis, Tennessee, describes its conclusions as "obvious", pointing out that the committee was "largely devoid of people practising clinical gene therapy".

As a result, Varmus "got the answer that he wanted" says Brenner, who claims that there is no "absurd bias" toward clinical research in gene therapy.

Nevertheless the report appears to vindicate the NIH's current strategy. In the financial year that ended in September, the institutes spent \$181.5 million of their \$11.3 billion budget on gene therapy, with about \$50 million going to basic research and the rest to clinical and applied research. It expects to spend \$194 million in the current financial year.

The panel described these spending levels as "appropriate". It did not recommend establishing special gene therapy study sections, as clinicians had urged. "Future gene therapy research should compete with other forms of biomedical research for funding under stringent peer review," the panel reported.

Meredith Wadman

Funds are key to sequence success

Washington. Francis S. Collins, director of the US National Center for Human Genome Research, predicted last week that, "barring a disaster in funding", 99 per cent of the human genome sequence will be completed at an accuracy of 99.9 per cent by the year 2002 or 2003, considerably earlier than the initial target date of 2005.

At the same time, he expressed both concern and frustration about a continuing dispute over the NIH budget. Collins pointed out that, more than two months into the current fiscal year, he still does not know the size of the project's budget for the year. As a result, his office is reviewing proposals for large-scale sequencing work "not knowing if we have any money to spend".

Collins was speaking at a symposium held to mark the fifth anniversary of the Human Genome Project. He pointed out that, for a number of goals set in 1990, "we

are either ahead of schedule, under budget, or both". For example, linkage maps due to become available at the end of 1995 were published in mid-1993, and high-accuracy physical gene maps are now expected to be available in 1998.

He confirmed his view that the time is ripe to "shift full tilt into sequencing" from mapping. In three years, Collins predicted, every genome centre funded by his office will be doing sequencing, compared to the handful now doing so.

Yet even though there is both bipartisan support in Congress and enthusiasm in the administration for human genome research, funding prospects remain uncertain. "When people are in a position of trying to make cuts wherever possible, and deals are being made late at night behind closed doors, you never know how it's going to settle," Collins said.

Patrick Young

Foresight wins bouquets and brickbats from watchdog

London. An all-party British parliamentary committee has sharply criticized the ways in which the government is implementing key recommendations of the white paper on science and technology, published in the summer of 1993.

The criticism comes in a report published last week by the House of Commons Select Committee on Science and Technology at the end of a three-month inquiry into the Technology Foresight programme, introduced by the government as a way of identifying strategic priorities for government spending on research.

The committee has high praise for the way the Foresight programme, which initially operated through 15 subject panels, was both conceived and put into practice. Indeed, it describes the completion of the first 'survey' stage of the programme, which involved polling both the academic and industrial research communities on future technological needs and opportunities, as "a remarkably impressive achievement".

"Technology Foresight is a good thing," says Gavin Shaw (Conservative, Pudsey), the chairman of the select committee. "We recognize that a tremendous amount of effort has gone into it, and the increase in awareness that has been the result."

Such comments reflect a widespread feeling in the scientific community that the programme, after a somewhat bumpy start, has turned out to be more effective in promoting dialogue (and less rigid in imposing priorities) than many had feared. A recent report from the Royal Society, for example, points to what many consider to be one of its main achievements, namely the creation of active 'networks' connecting researchers to those potentially interested in implementing their results.

But the select committee, which effectively works as a parliamentary watchdog on British science policy, is still concerned about aspects of the critical next stage, the way in which the results of the programme are being put into practice.

For example, it reflects the fears of committee members such as Anne Campbell (Labour, Cambridge) that the government might use the Technology Foresight exercise to determine the research councils' spending priorities and increase the pressure on funds for basic research.

The committee's sharpest remarks pick up on comments made by, among others, the National Farmers' Union, on the absence of any life scientists from the revised membership of the Technology Foresight steering group, the body responsible for overseeing the development and implementation of the programme.

Admitting that the steering group cannot duplicate the sector panels, the committee nevertheless points out that "such a lacuna may prevent it from carrying out its oversight role effectively". Noting, furthermore, that none of the industrialists on the group comes from an industry which is based on the life sciences, it suggests that "serious consideration be given to adding someone with expertise related to the sector underpinned by biological science".

Government officials reject this criticism. They point out that individuals are appointed to the group in their own right, not as representatives of a particular discipline or interest group, and that the life sciences are not entirely absent, as the group is chaired by Robert May, the government's chief scientific adviser, who is a population ecologist.

But Shaw describes such a response as "a bit glib". He adds: "There is concern that the life sciences, which are an important part of the science base, have come out as a loser in this. We can only say we have taken note of that concern, and must monitor the situation to see what happens in practice."

The committee also has some harsh comments about what it perceives as the failure of the Office of Science and Technology to exert a stronger role in coordinating research policy across different government departments. In particular, it focuses on shortcomings in the publication of the *Forward Look*, an annual document on total government spending for research and development, promoted as the main vehicle for exercising such influence.

Referring, for example, to the lack of detail on topics such as the long-term science plans for various government departments, the committee says "this seems to be evidence that the transdepartmental co-ordination promised in the 1993 white paper is not working satisfactorily".

The government will, in due course, provide a detailed response to the select committee's comments. While accepting some of the detailed criticism concerning the lack of figures in the *Forward Look*, it is expected to defend itself against the broader charges, which many see as reflecting a long-held desire by the select committee for a more powerful role for science in Whitehall than other politicians may be prepared to accept.

David Dickson

East German scheme fails to rehabilitate university scientists

Munich. Only one in 20 of the 2,000 scientists from the former east German Academy of Sciences who had been promised permanent jobs in universities have actually been offered such positions, according to a survey conducted by the university teachers union, the GEW.

The researchers involved are those who had been earmarked for university posts by the *Wissenschaftsrat*, Germany's science council. The fate of the remaining scientists remains unclear, as the *Bund-Länder Kommission* (BLK), the body that mediates on research and education between federal and *Länder* governments, voted this week to delay until next spring any decision on new programmes that could provide further temporary support.

The scientists had been chosen from the thousands who lost their jobs when their research institutes or departments were closed after the reunification of Germany. The *Wissenschaftsrat* thought that transferring them to universities would help to solve a second post-reunification problem, namely the reintroduction into universities of research, which had been effectively banished into academy institutes during the communist years.

As a result, the federal and *Länder* governments jointly set up a five-year, DM600-million (US\$414-million) programme, known as the *Wissenschaftler-Integrationsprogramm* (WIP), to support the scientists while they found new positions.

But the programme, which runs out next year, has not worked. The universities had already been forced to dismiss many of their own staff as a result of the 'restructuring' process introduced after reunification, and were reluctant to take on 'outsiders'. About a quarter of those originally recruited into the WIP scheme dropped out, and most of the rest now have no hope of a university position. The federal government continues to insist that the WIP programme will not be extended.

At a meeting organized by the GEW in Berlin last week, the governments of four of the new *Länder* announced that they would provide additional short-term research funds to support their own WIP scientists beyond 1996. Gerd Köhler, a member of the GEW committee for universities and research, said the union would monitor the schemes to ensure that all WIP scientists continued to be suitably employed.

But the union is not insisting that WIP scientists be integrated into universities. That demand has been made only by Berlin, where more than a third of all WIP scientists are employed, and which has the most severe financial ►

IMAGE
UNAVAILABLE
FOR
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REASONS

Campbell: seeking to protect basic research.

Japan's Diet eyes technology assessment

Tokyo. A committee with broad powers to analyse government science policy and provide advice to lawmakers on science and technology is to be set up in Japan if a bill to be put before the Diet, Japan's Parliament, early next year is approved.

The bill has been proposed by Taro Nakayama of the Liberal Democratic Party (LDP). The proposed committee — tentatively called the Science and Technology Assessment Committee — would be made up of 15 Diet members from all political parties, each of whom would serve a three-year term, supported by an office staffed by an estimated 105 researchers.

This office is expected to have a budget and management structure modelled on that of the US Office of Technology Assessment (OTA) which, ironically, was abolished by Congress earlier this year after 22 years (see *Nature* 376, 541; 1995).

Nakayama's proposal comes at a time when mounting political support for science and technology, spurred by continuing recession and fears that Japan may be left behind in key areas, has already resulted in a flurry of new legislation and other measures designed to bolster the country's position in high technology.

Nakayama himself is a qualified medical doctor and a former diplomat. He proposed the new bill as a representative of the Diet's Science, Technology and Policy Committee. He and others say the new assessment committee is needed because of the increasing scale and complexity of government-supported science projects, and the lack of a body capable of assessing their benefits and effectively coordinating the expenditure of public funds on research and development.

Nakayama, for example, wanted to cut funding for Japan's nuclear-powered ship *Mutsu*, which consumed more than a billion dollars of taxpayers' money over nearly two decades yet spent only a few days at sea. But there was no adequate mechanism for the Diet to cut off funding, while the Science

and Technology Agency continued to pour money into the project as it did not want to lose the budget.

Details of the proposed committee's priorities have not been made public. But areas within its remit are expected to include the nation's space and faster breeder reactor programmes which some believe have not been adequately assessed.

According to one of his political colleagues, Nakayama feels that Japan is wasting a lot of money on the H-2 rocket, which costs twice as much as launch vehicles elsewhere. He thinks Japan should consider using cheaper Chinese or Russian rockets to launch Japanese satellites.

The latest proposal follows last month's approval of a law on basic science and technology passed by the Diet in the belief that the government should do more to support basic research, particularly that which may sow the seeds of future industrial technologies in areas such as materials and biotechnology (see *Nature*, 378, 227; 1995).

Nakayama originally wanted to pass both the basic science law and the present bill as a package, and is said to have been extremely angry when the basic science law was passed first. Both this law and the latest proposal originated as private members' bills drafted by members of the LDP, some of whose younger members in particular appears to be keen supporters of science and technology (see *Nature* 378, 8; 1995).

The main body responsible for providing the Japanese cabinet with advice on science and technology at present is the Science and Technology Council. This is a mixture of academics and politicians, and includes the ministers from all government departments and agencies responsible for supporting science, chaired by the prime minister.

But although the membership of these ministers and the prime minister lends the group an apparent authority, many inside and outside the government feel the council is not as influential as its prestige suggests.

Stephen Barker

UK geophysicist killed in San Diego

San Diego. Keith Runcorn, a British geophysicist who was widely known for his work on continental drift, was strangled in his hotel room in San Diego, California, last week while on a lecture tour. San Diego police have made no arrests in the murder of Runcorn, who held an endowed chair at the University of Alaska in Fairbanks.

In the 1950s, Runcorn was an early proponent of the theory that continents slowly drift apart, gradually altering the map of the world. Plate tectonics, as the theory became known, revolutionized geology, spurring him and other geophysicists to debate for decades the merits of the concept.

Runcorn later worked with Patrick (later Lord) Blackett at the University of Manchester, before returning to the University of Cambridge. He also carried out research

on remnant magnetism, at the University of Newcastle upon Tyne.

A scientist who thrived on controversy, the red-haired Englishman was a member of the committee that monitored Biosphere 2, the experimental habitat near Tucson, Arizona, that doubles up as both a research project and a tourist attraction. He was meeting academic colleagues in San Diego, Los Angeles and San Francisco on the trip when he was killed.

Runcorn was staying in downtown San Diego, in a hotel which is surrounded by new office towers and plush hotels with stunning views of San Diego Bay. According to police, he had checked into the older, refurbished hotel in order to save money and be near restaurants and shops, and had planned to stay there a week.

Police said he had a blunt-trauma wound to the head, and had also been strangled. Runcorn's room was partially ransacked, and he is thought to have been dead for a number of hours before being found.

The neighbourhood is not known for serious crime, and tourists walk the area at all hours. Murders of such prominent individuals are rare in San Diego, where most killings are related to gangs or the drug trade the flourishes near the border with Mexico.

Rex Dalton



Runcorn: thrived on scientific controversy.

► problems of all the new *Länder*.

Manfred Erhardt, the city's research minister, told the meeting that the WIP programme should be extended to buy more time to achieve the original intention of genuinely integrating the scientists into universities. The University Rectors' Conference (HRK) supports this position, but has no direct say in decision-making.

Some new money earmarked for east German researchers, including WIP scientists, could be made available if plans come to fruition for a third *Hochschulsonderprogramm*, a trouble-shooting programme aimed at solving particular university-related problems, such as the low representation of women in academic institutions.

In principle, all sides agree that such a

programme is needed, particularly to help iron out inequalities between east and west Germany. But at its meeting this week, the BLK was unable to decide how much money the programme should have, and how the bill should be split between federal and *Länder* governments. It therefore delayed a decision until next March.

The federal government would like the total cost to be DM3.6 billion and wants the *Länder* to pay 60 per cent, but the *Länder* would like a total of DM4.1 billion, split 50:50. Moreover, the federal government argued that the final decision will depend on the outcome of other contentious issues, such as how much money it will have to spend on student grants and university buildings.

Alison Abbott

Speaking ill of the Dead

SIR — I was disappointed by John L. Casti's remarks about the rock band "The Grateful Dead" in his review of Tyler Volk's *Metapatterns: Across Space, Time, and Mind* (*Nature* 377, 24; 1995).

Jerry Garcia, the band's lead guitarist, was no lightweight entertainer. On the contrary, his career has been likened to that of jazz greats such as Louis Armstrong, Miles Davis and Charles Mingus. "The Grateful Dead" were not only engaged with the real issues of our time but practically married to a philosophy of change through a very simple musical model. The beauty inherent to their wizardry was often astounding to behold, physically and spiritually. In addition to the musical magic they created for the pleasure of millions, the members of the band were socially conscious. They participated in countless benefits to fight homelessness, rape, AIDS, environmental issues and blindness, and helped to promote the handicapped and education. They were genuinely interested in preventing and relieving suffering and promoting hope through compassionate action.

Among other projects, their participation and influence helped to provide support for: more than 30,000 cataract operations and the building a state-of-the-art eye hospital in the Lumbini zone of Nepal; more than 200,000 surgical operations at Aravind Eye Hospital in Madurai, India; nutrition, health and education programmes at three refugee camps in Mexico; the creation of the Jaguar Project to accompany refugees returning to Guatemala; devastated Guatemalan villages by supplying animals, tools and seeds to help reduce their dependence on imported fertilizers; Mayan farmers for community development projects such as housing, water supplies, health care and cottage industries; grassroots organizations on Native American reservations to provide health clinics, protect the environment and safeguard traditional knowledge; a range of grants to help preserve Tibetan culture.

Their accomplishments will ensure that they are remembered far beyond the life of Jerry Garcia and the musical life of "The Grateful Dead".

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Pain pathways

SIR — You recently published two Letters^{1,2} reporting a new ATP receptor on neurons in the pain pathway. I should like to expand on the brief reference to the historical background given in the accompanying News and Views article³. The papers published in the 1950s by Francis and the late Pamela Holton^{4,5} gave good evidence that the transmitter for antidromic vasodilatation is ATP.

It was then already well established (see, for example, Thomas Lewis's classic of 1927⁶) that this phenomenon, blood vessel dilatation in an area of skin when the sensory nerve to that area is stimulated, is the efferent side of the axon reflex, the dilatation of skin vessels resulting from activation of pain fibres in the skin and conducted to nearby blood vessels through branches of those fibres. So the connection between ATP and pain goes back more than 40 years and is not a new idea as implied in the News and Views article. It is also depressing that authoritative works of 1971 and 1984 stated that the transmitter for antidromic vasodilatation was unknown^{7,8}.

The motive given by the Holtons for setting out to identify the peripheral transmitter of these pain fibres was that it would provide a suggestion for the identity of the peripheral transmitter used at the central end of the same neurons in the spinal cord or brain stem, on the basis of the proposal by Henry Dale⁹ in 1935 that a nerve cell was likely to use the same transmitter at both its peripheral and its central terminations. This farsighted plan by the Holtons links their work even more closely with the discovery of appropriate receptors on nerve cell bodies in the pain pathway. It is satisfying that their surmise, well known at the time, has at long last been given further support; sadly, Pamela Holton died at an early age in 1977 and never knew how accurately she and her husband had foreshadowed this discovery.

Andrew Huxley

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Security at MIT

SIR — Your News story (*Nature* 378, 9; 1995) about a researcher's ingestion of phosphorus-32 at MIT, which quotes a statement by me, implies that I was aware of a security problem involving an open

door in that laboratory. So far as I know, security practices there did not differ from practices in other laboratories at MIT where similar research was conducted. The incident to which I referred was in an entirely different MIT building. In addition, contrary to your article's wording, the door in question was only temporarily, not permanently, kept open, and keeping that door open was contrary to MIT policy, not to any requirement of law.

Kenneth D. Campbell

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Colonial dinosaur

SIR — Kipling, had he been alive, would be glad to know that the fawning, loyal subject of colonial days looking up to his overlord for justice is not extinct. B. N. Dwivedi's letter (*Nature* 376, 545; 1995) shows the same sovereign contempt and distrust in which the colonial subject held his compatriots and the same eagerness to go crying and complaining about supposed wrongs to his master and cling to his garment.

Using the columns of *Nature* as a vehicle for self-praise and denigration of this country, this writer expects to be patted on the back by announcing his admiration for Oxford, Cambridge and Glasgow, and, perhaps, also expects to be invited to enjoy spells of "home weather".

Meanwhile, scientists and professors in India are supposedly employed in impeding not only the progress of science but also that of "conscientious" persons like your correspondent who "loves to teach but seeks to be evaluated on his research work".

Young engineering graduates, according to him, must not encroach upon physics, although an exception can be made in the case of Dirac because of his association with Cambridge.

The Indian science community is allegedly drawing inspiration from the members of one of the world's oldest professions. Will they be grateful for this dubious distinction conferred on them? They may have better decency and judgement.

The first impulse of many readers of Dwivedi's letter may be to condemn it to the oblivion it deserves, but the Indian Department of Science, the government, the national laboratories, institutes and universities and all Indians will have to take note of this denigration of India and Indian science in journals published abroad. Indian science and its administration are, unfortunately for Dwivedi, no longer the "white man's burden".

S. S. Kushwaha

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Survival of Italian biomedical research

Jacopo Meldolesi

Despite crises in image and funding, Italian biomedical research has reached a turning point. A brighter future is in prospect if the universities successfully reorganize themselves and new competitive institutes are established.

THE Italian university system has suddenly come into the limelight. Over the past few weeks, national newspapers have devoted whole pages to exposing the latest of Italy's corruption scandals. Essentially, two aspects are now under scrutiny: the ethical status of professors appointed because of their personal connections rather than scientific merits; and the painful status of students, whose misfortune is to be taught by inappropriately qualified and often absent professors. But in addition to its immediate effect on students, this complex situation threatens the future of the country as a whole, because university scholars play an essential part in the development of science and technology as well as in teaching. In this respect, the state of biomedical research is now particularly vulnerable.

During the past few decades, Italian biomedical research has attracted the attention of the international community mainly because of negative aspects such as the low level and poor relevance of public investment in the field (less than half the average of the other main European countries); the questionable appointment criteria for university positions; the scarcity of Italian publications in leading journals and of Italian scientists in international committees and laboratories (such as the European Molecular Biology Laboratory); and the emigration of Italian scientists of all ages to other European countries and to the United States and the lack of effective policies to attract them back home. Yet the scientific potential of Italy, based on scientists still active there and the many others interested in returning to the country, remains considerable.

The situation has recently deteriorated even further, especially at the level of initiatives taken by the universities and the National Research Council (CNR). Devaluation of the currency (more than 30 per cent against the US dollar and the Deutschmark) has been accompanied by a decrease in research funding rather than a compensatory increase. In addition, much of what investment remains has been oriented, at least officially, towards applied rather than basic research. Examples are the so-called 'target projects' that have dominated CNR biomedical policy during the past 15 years; the (bureaucratic and inefficient) projects of the Ministry of

Research, which are addressed to industry and which recruit laboratories in universities and other institutions only as 'third parties'; and the recent decision of the Conferenza dei Rettori (the Committee of University Rectors) to favour applied research in university laboratories. The reduced impact of public support cannot be compensated for by the drug companies. Because of poor scientific standards



University of Milan: will it break with tradition?

and vast corruption, the national drug industry has almost disappeared, and Italy has an important research role only in a few of the multinational pharmaceutical corporations such as Pharmacia, Glaxo and Merck.

Despite the gloom, a few changes have started to help good research to develop in Italy. Steps have been taken by the Istituto Superiore di Sanità, the technical body of the Ministry of Health, to improve the public grant system. Three new medically oriented programmes have been begun, with improved criteria and with evaluation procedures based on peer review: the AIDS programme is now in its eighth year, and the tuberculosis and the multiple sclerosis programmes began this year.

Private charitable sources are also showing promising signs. The Telethon Foundation has widened its interests from muscular dystrophy and other neuromuscular diseases to include all genetic diseases and has opened an advanced research institute (the Telethon Institute of Genetics and Medicine) in Milan; its grant evaluation system has been

improved and is now based on peer review controlled by a qualified international committee. AIRC, a private charity supporting not only clinical but also biological research in cancer research, has become more prominent, while the funding of another private charity, AISM, which supports research into multiple sclerosis, has recently been expanded.

The long-term effects of the European Union's biotechnology and biomedical programmes may be even more profound. Although specific aspects of these programmes have been criticized, on the whole they have had a beneficial role. In particular, the participation of Italian research groups in high-quality international projects, a necessary criterion for the submission of successful applications to these programmes, represents a new evaluation standard in Italian research and provides young scientists with an opportunity to broaden their research experience and improve their qualifications.

Collectively, these changes have greatly altered the outlook for biomedical research in Italy. Although the official policy of public support agencies for science (the CNR, universities and so on) has remained unchanged, their role has decreased considerably, in most cases hardly serving beyond merely allowing an institution to survive. In other words, although the so-called 'rain support' continues, with drops reaching essentially all laboratories doing any research, the drops have often shrunk below any operational value. But new initiatives at the level of laboratories, departments and institutes have meanwhile gained substantial support from more demanding funding agencies, and their potential is likely to continue to increase in the near future.

On the basis of these experiences, the need to modify traditional research management in Italy has become increasingly clear. To be productive, the changes should not be based only on examples from other countries. They should draw on the experience of those Italian institutes, new and old, where appropriate policies have been developed and where a 'critical scientific mass' has therefore been reached as a result of equal relationships established with qualified foreign groups.

There are several changes that,

although not easy to make, seem possible on the basis of the present situation and which, in the years ahead, could have an increasingly beneficial influence on the progress of biomedical research in Italy.

Take, for example, the change of status of the universities. In Italy almost all universities belong to a national system. Until two years ago, their organization was strictly centralized and the scope for local initiatives was limited. A new law has changed this state of affairs by requiring universities to transform themselves into public enterprises.

In practice, the effects have so far been minor: altering the spirit and operation of established institutions is difficult, and most of Italy's principal universities are huge (with tens of thousands of students) and their functioning reflects a fine balance of many varied interests. But at least some of the interested parties might quickly be attracted by the new economic picture and so begin to realize how advantageous it would be to have ample access to grants. From this standpoint, good scientists could become essential components of a pragmatic strategy that would make available new opportunities to universities in terms of funding as well as image and international interactions.

Precise criteria

This realization will have far-reaching consequences, even affecting access to research and university posts, an area that until now has been regarded by the Italian academic establishment as its sole preserve. Scientists would have to be chosen exclusively on the basis of precise — and internationally accepted — scientific criteria. Moreover, individual universities will have to understand (much better than the Ministry of Research) that competitive scientists need not only a salary but also adequate working conditions, including laboratories, facilities and the 'critical scientific mass'. They could therefore sponsor — or participate in — the development of new institutes funded not on the previous criteria (such as over-narrowly defined academic disciplines and membership of specific scientific schools) but solely on the quality of scientists and their productivity (research results, grants and so on). In these institutes the participation of universities could enhance the progress of basic research, which would develop together with the more applied studies supported by the 'scientific hospitals' of the Ministry of Health, charities and the European Union.

Examples of such institutes already exist, having been established over the past 5 years by private foundations and by non-university consortia. The DIBIT of the San Raffaele Institute in Milan, where I am scientific director, has a staff of 80 scientists, ten of whom hold permanent university positions. Another institute, the

CBA, has been conceived with the participation of a university and serves as the biotechnological branch of the Cancer Institute in Genoa. Two other cancer research institutes have been or are about to be established: the EIO in Milan, and the IRCC in Turin. Mario Negri in Milan, a foundation that has been active in pharmacology for some three decades, has opened a new institute near Pescara (east of Rome). Even Merck's research institute south of Rome has connections with universities.

There now need to be more institutes of this kind, maintaining or even improving the quality-based criteria on which they have been developed. This is not a bizarre idea, valid only for Italy. In fact, new highly respected institutes have recently been, or are being, developed (or remodelled) in other advanced European countries, such as those of the Medical Research Council and the Wellcome Trust in the United Kingdom, the new Max Plank Institutes in Germany and the Marie Curie Institute in Paris. Although some may think that similarly rapid development on this scale is unrealistic in Italy, I am convinced of its possibility. Once one university takes the initiative, others could quickly follow suit.

A second area of change concerns the interaction between basic and industrial biomedical research. Traditionally, these two worlds have remained almost completely separate, with individual professors mediating interaction with the scientific world as well as the centralized health authorities according to his or her personal prestige and knowledge in the field. By contrast, the new model of interaction is based on the coexistence of basic and industrial research institutes in appropriate science parks. Again, this initiative is not new but has already been successfully applied in other countries. In fact it answers precise needs that have recently arisen from scientific progress that demands highly qualified and multidisciplinary expertise. Unless carried out in major independent institutes, where the 'critical scientific mass' can be reached, the industrial research needs to be nurtured in a favourable basic-research environment where fruitful collaborations can be established and where the right facilities are available.

In addition to the obvious cultural and research advantages, initiatives of this kind can ultimately provide bonuses for both basic and industrial research: for basic research they can provide money for the acquisition of expensive facilities, whereas for industrial research they can improve the general working environment and attract graduate students and postdoctoral researchers otherwise excluded from industrial laboratories. Such continuous interaction can also encourage staff exchange and thus a

healthy degree of turnover among researchers. Initiatives of this type (for example in the San Raffaele Biomedical Science Park, next to DIBIT in Milan) are new to Italy and promise to yield further important developments.

Political commitment

Yet what changes have already taken place are destined to be little more than one-off episodes as long as Italy's political environment remains largely uninterested in science and its future. The reorganization of universities, with the development of new competitive institutes, in fact requires new rules to assure flexibility in management, rigorous procedures in the selection of scientists and quality control of the work carried out. Progress is slow, and this is partly understandable. The policy turmoil in Italy during the past two years and the severe economic crisis have yielded a technical, non-political government that is expected to last for only a relatively short time and which is necessarily preoccupied with various emergencies. Despite the estimable qualities of Giorgio Salvini, the minister in charge of universities and research, an eminent physicist and the former president of the *Accademia dei Lincei*, further cuts in funding for research have been proposed in the finance bill now being discussed by parliament. But the election law introduced two years ago was intended to ensure stable (ideally five-year) governments. After the general election, which will probably be held next spring, the new government (no matter whether centre-left or centre-right) will no longer be able to avoid giving serious answers to essential questions, including whether Italy wants an internationally significant role in drug development, biotechnology and medicine.

Needless to say, successful growth of applied research depends on original methods and ideas developed by basic research. So investment in basic biomedical research and an efficient policy for reversing the 'brain drain' should not be postponed. Inasmuch as private foundations support primarily medical research, these tasks should be undertaken by public agencies, in particular by the CNR, which should be drastically reorganized. This implies a complete reversal of the policy implemented by Italian public agencies during the past few years, which has focused mainly on applied research — a shift that, it is to be hoped, will keep many of us busy outside our strictly experimental time, and in which we hope to be able to count not only on sympathy but also concrete support from the international community. □

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A new phylum from the lobster's lips

Simon Conway Morris

On the taxonomic hierarchy that runs from species to kingdom, phylum ranks one level from the top. There are only 35 or so animal phyla, to which the Cycliophora must now be added.

MILLIONS of species remain undescribed, and given the present rates of habitat destruction a good number will probably say farewell to the Earth under a bulldozer or pile of sludge. Nevertheless, the general rule is that wherever the zoologist looks, from the abyssal depths of an oceanic trench to the canopy of a tropical rainforest, the species may well be new to science, but the major groups to which they belong will almost certainly be well known. This is particularly true of the 35 or so basic body plans (or phyla) of animals. With only a few months' training a zoologist staring into a bucketful of deep-sea worms should have few difficulties: "That's a nematode; careful with that annelid, it bites; I never knew sipunculans were so slimy...". To report, as Funch and Kristensen do on page 711 of this issue¹, what appears to be a new phylum of metazoans has to be the zoological highlight of the decade.

What makes this discovery even more astonishing is that the representative of the Cycliophora, as they term the phylum, was not collected on the tops of a remote mountain range in Central Asia or beside a deep-sea vent, but attached to the mouthparts and appendages of the Norwegian lobster, including specimens collected in the Kattegat straits, between the northern tip of Denmark and Sweden. This new animal, named *Symbion pandora*, is therefore epizoid and also small, being less than a millimetre in length. Its anatomy is vaguely reminiscent of several phyla, including the relatively obscure entoprocts, as well as the ectoprocts (or bryozoans) and rotifers. In essence, the body is sac-like, adhering to the host lobster by an adhesive disk. An anterior funnel with a ring of cilia sweeps food particles into the mouth. The gut itself is recurved, so that the anus opens close to the mouth, on the upper part of the trunk (Fig. 1).

In the tradition of the best scientific papers, the announcement of the Cycliophora raises many more questions than it answers. Take, for example, the complex life cycle. The presence of a dwarf male hanging onto the relatively gigantic

female-bearing feeding stage certainly finds parallels, such as in some echiuran worms and perhaps most famously in the deep-water angler fish. The presence of both asexual and sexual cycles again finds parallels elsewhere in the metazoans, but the course of the asexual cycle in *Symbion*, whereby there is a series of interior buddings associated with autolysis and total

of ignorance of the group's wider systematic position — it must be more closely related to one group or another. Nevertheless, at the moment the way forward is far from clear. The idea of entoprocts and ectoprocts being closely related has been vigorously championed by Nielsen², but so far it has not won very wide acceptance³. As far as I am

aware, no molecular evidence that might help to elucidate the position of entoprocts is yet available. In terms of the ectoprocts this line of enquiry, specifically the study of ribosomal DNA, generally upholds the traditional view of their being related to the lophophorate phyla of brachiopods and phoronids^{4,5}. With the anatomical arguments apparently exhausted, molecular evidence may be crucial in determining whether entoprocts and ectoprocts are close relatives. And this applies with equal force to future investigations into the Cycliophora. Even if this new phylum is not an actual entoproct, could it be some sort of progenetic relative (that is, one subject to precocious sexual maturation)?

In this context it might be worth remembering that this discovery is not the first new phylum to be found by R. M. Kristensen. Some years ago he reported an intriguing new group of animals, again microscopic, known as the Loricifera⁶. In this case, however, its relationships with other phyla, notably the priapulans and kinorhynchans, are fairly clear². Some workers, however, have gone further to suggest that the loriciferans may simply be neotenic priapulans⁷ (in other words, priapulans that have a reduced rate of morphological development during the juvenile stage). At the moment the Cycliophora do not appear easy to

squeeze into either a progenetic or neotenic mould, although at least in the former case the emphasis on a ciliary feeding system and conceivably loss of organs such as protonephridia are plausible consequences of such miniaturization.

In current parlance *Symbion* belongs to a sort of superphylum (or perhaps superdustbin) known as the Aschelminthes. Both the inter-relationships of the seven or so constituent phyla, of which the

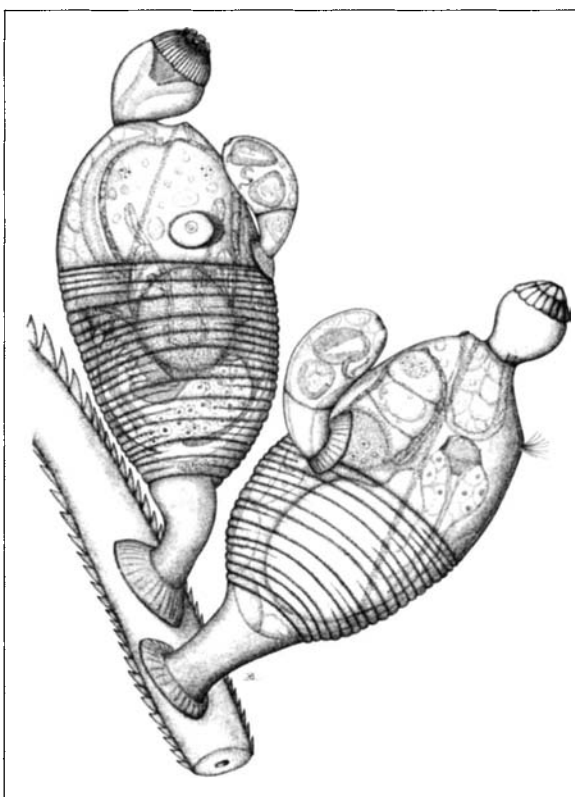


FIG. 1 Two feeding-stage individuals of the cycliophoran *Symbion pandora* at a crucial moment in their intricate sexual cycle; each is some 350 μm in length, and feeding parts are located at the top of the trunk. Tiny males are attached to the outer surfaces, while females wait inside. The females will eventually escape, disperse and develop into 'chordoid' larvae, which settle on a new host and initiate an asexual cycle of budding and release of a second type of larva, the 'pandora'. See page 714 for more details.

regeneration of the internal organs, culminating in the ultimate release of the so-called pandora larva, is strange indeed. The initiation of the sexual cycle seems to be connected with the onset of the moulting cycle of the lobster. One wonders what chemical cues, perhaps from the host's hormones, are used.

Even if the Cycliophora win acceptance as a new phylum — and never forget that this taxonomic rank is in part an admission

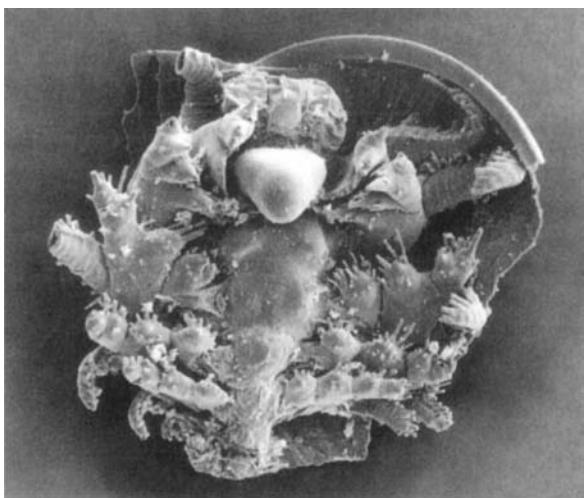


FIG. 2 The Upper Cambrian arthropod *Hesselndona* n. sp. recovered by acid digestion of limestones (Orsten) from Västergötland, Sweden; the specimen is about 510 million years old. This ventral view shows the remarkable quality of the fossil preservation by phosphatization. The fine setae of some of the appendages are comparable in size to those of the Norwegian lobster to which the Recent *Symbion pandora* has been found attached. The possibility of finding fossil representatives of the Cyclophora cannot yet be ruled out. (Photograph ($\times 150$) courtesy of K. J. Müller, Institut für Paläontologie, Univ. Bonn, Germany.)

nematodes are probably the best known, and their overall place in the metazoan tree⁸ are very controversial. To be sure some links are beginning to

look more secure. Thus, present evidence suggests that the lake-dwelling rotifers and the acanthocephalans, which as adults are endoparasites in the guts of vertebrates, are actually quite closely related⁹. Reasonably, Funch and Kristensen¹ consider whether *Symbion* is related to the rotifers, but they conclude that the evidence is hardly compelling.

When the Cyclophora first appeared in the geological record is, of course, entirely conjectural. We should not, however, rule out the possibility of finding fossil representatives. For all we know cyclophorans may attach themselves to a variety of substrates, but the recovery of superbly preserved arthropod limbs complete with fine setae from the Cambrian^{10,11} (Fig. 2) suggests that ancestors of

Symbion might yet be recognized.

This report leaves one wondering how many other phyla may have been overlooked. Even now there are several very

peculiar animals, such as the lobatocerebrids and xenoturbellids, which apparently defy placement in known metazoan groups¹². Next time you are at your favourite seafood restaurant, make sure the waiter has to hand a couple of zoology textbooks and a decent microscope on the pudding trolley. Who knows what might be found lurking under the lettuce? □

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SYNTHETIC CHEMISTRY

In support of the pore

Stephen Lee

IN 1756, the first zeolite was discovered. These proved to be inorganic minerals which, when heated, released water but retained their original crystalline form. The heating process had led to a porous solid with cavities the dimensions of ordinary molecules. Since zeolites were first discovered, these inorganic aluminosilicates and aluminophosphates have found ever increasing usage both in catalysis and in storage. On page 703 of this issue, Yaghi, Li and Li¹ now report that they have synthesized a solid with this same porous capability, but with a primarily organic framework that could offer much more flexibility in pore size and functionality.

To prepare this organic analogue to zeolites, Yaghi *et al.* initially needed an organic low-density solid. To create such a material, they noted that solids with only a small number of bonds between the constituent atoms are generally the least dense. Examples of such low-density solids are graphite and diamond, where the carbon atoms have respectively three and four bonds to each atom. As others have done previously^{2–5}, the authors

therefore chose a trigonally symmetric molecule, in this case 1,3,5-benzenetricarboxylate (btc), as a molecular analogue to the three-coordinate planar carbon atom in graphite. This molecule, when co-crystallized with cobalt nitrate and pyridine, did indeed form a structure analogous to the honeycomb layers of the graphite structure. But because btc is much larger than individual carbon atoms, the resultant structure has much larger pores than those found in graphite itself (its holes are 7–10 Å in length).

The authors then found that when they heated this material to 200 °C, a proportion of the co-crystallized pyridine solvent molecules could be boiled away without loss of the original crystalline structure. In previous work, similar low-density organic solids have exhibited the ability to exchange one solvent for another^{3,6}, but this is one of the first instances where a solvent has been removed without another being substituted⁷. On further heating to 350 °C, all the pyridine can be removed, but at this point the crystal structure collapses into another, presumably denser phase. In follow-up experi-

ments which are likely to stimulate a flurry of further research, Yaghi and co-workers found that the less dense porous solid selectively absorbs aromatic rather than non-aromatic solvents.

Although it is difficult to pinpoint why the new compound is able to withstand the partial loss of solvent, certainly a crucial factor in its overall stability must be the strength of the remaining interactions in the solid: the cobalt–oxygen coordination bonds and the π – π stacking between adjacent aromatic layers. What is clear is that the primarily organic extended solid made by Yaghi *et al.* is one of the first that can successfully mimic the porous nature of the original zeolite compositions.

One of the best known applications of inorganic zeolites is the Mobil process which uses the zeolite ZSM-5 in the production of petroleum from methanol. Do porous organic solids have an equally bright future? This question touches on one of the central issues in synthetic chemistry — whether reactions are best carried out in homogeneous solvents rather than heterogeneous media. One might argue that they are not: in solvents, after all, reactions are governed by stochastic processes, so there is insufficient control of the reaction transition state. Much better would be the exact control of the reaction environment so that one could tailor a reaction to a desired out-

come. Certainly in our own bodies considerable effort is expended on the production of proteins for exactly this purpose. The hope is that 'designer' porous compounds such as that constructed by Yaghi *et al.* could offer the same advantages in synthetic chemistry.

But the idea has proved hard to carry out in practice. One need only enter a modern synthetic chemistry laboratory to see how little weight such arguments really have. Unlike laboratories devoted to most other fields of natural science, today's synthetic chemistry laboratories often look much the same as they did a century ago; they are filled with flasks, heating elements and above all solvents, the effective medium of almost all reactions.

One might speculate why chemistry laboratories have been so little affected by existing inorganic porous solids. One possible explanation is that these inorganic porous solids bear little chemical resemblance to the organic solvents with which most synthetic chemists are comfortable. Silicates and aluminophosphates are more akin to the glass flasks than to any organic solvent. The porous solid reported by Yaghi *et al.* might represent a bridge between these two chemical worlds. The building block that they use, trimesic acid, is a lot like benzene itself. Just like an ordinary solvent it accepts certain functional groups into its pores and not others.

Of course, the new compound is just a first step in this direction¹. Considerable obstacles lie ahead, and bulking large among these is the current use of bonds between carboxylates and transition metals rather than the stronger covalent bonds. Carrying out a reaction in such a medium would be rather like carrying out a reaction at one end of an organic molecule while there is an even more reactive site on the opposite side. Sometimes the reaction will be successful but all too often it will fail. It may prove possible in the future to fashion even stronger organic porous networks which will obviate such concerns. The outlook is one of both challenge and promise. □

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Trees under tension

Ernst Steudle

If a non-botanist is asked how water moves up to the top of a tree, the answer will probably be that it is the result of capillary forces acting in the narrow vessels that run vertically up the plant (collectively known as xylem). But this answer would be wrong. Diameters of xylem vessels range from 10 to 20 μm in conifers, to some 100 μm in broad-leaved woody species, which is far too wide to allow any substantial rise of water simply by capillar-

According to the cohesion theory, water is pulled upwards by the tension in xylem conduits that is created by the evaporation of water from leaf cell walls. If tensions are larger than one bar, pressures within the xylem conduits of transpiring plants are negative, that is, they are less than a vacuum. Negative pressures as low as -100 bar have been estimated in the xylem.

Because no metabolic energy has to be expended to drive water from the soil into the shoot and eventually into the atmosphere, the cohesion mechanism is of some advantage to the plant. Nutrients are cheaply transported in the transpiration stream as well. Water flow is driven by the difference in free energy of water in the soil and dry air (the difference in water potential). Usually the saturation deficit of the atmosphere represents a substantial force which is sufficient to create large tensions in the stiff xylem conduits. As transpiration is actively regulated by the plant's leaves through the opening and closing of their pores (stomata), tensions in the xylem are controlled as well and tuned to the demands of the shoot for water.

Although the cohesion theory is well established, two problems have stood in the way of its complete acceptance. One is that water under tension (negative pressure) represents a metastable state comparable with that of an overheated or undercooled liquid. Depending on the rate at which small bubbles of water vapour (gas nuclei) develop, the system will cavitate and water vapour will be formed in equilibrium with liquid water. Under these conditions, the pressure assumes that of the equilibrium water vapour, which is definitely positive (for example, 23 mbar at 20 °C).

The other problem is a technical one, in that negative pressures are hard to measure directly in xylem vessels. The introduction of sensors into vessels under tension tends to cause cavitation as the xylem wall is ruptured, a tendency that increases with increasing tension. That is why nearly all of the measurements made on pressure in vessels are indirect, as in the commonly used pressure chamber technique⁴.

Because of the difficulty of proving the existence of high tensions, the cohesion theory has occasionally been questioned^{5,6} and osmotic mechanisms have been proposed as an alternative explanation. Active uptake of nutrient salts into the root xylem creates a high internal osmotic concentration and water flows into the root. This in turn causes a positive root pressure, so instead of being sucked into the shoot, xylem sap is pushed upwards.

Hydraulic high — in trees such as these redwoods water has to be moved upwards by 100 m or more.

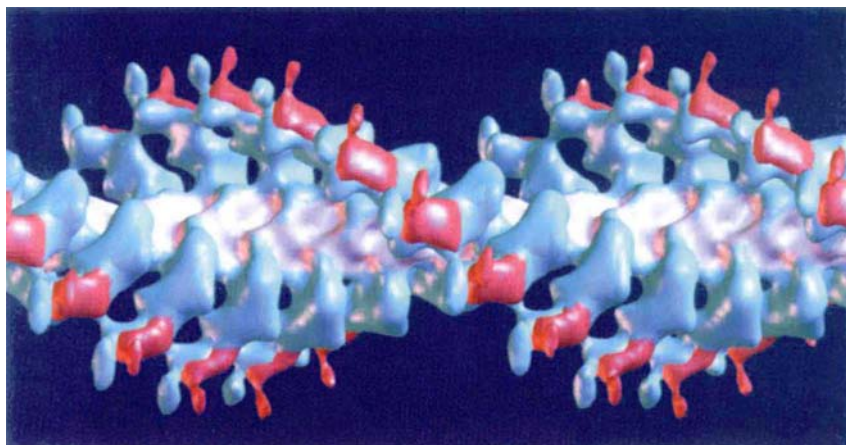
ity ('adhesion'). For example, a 10- μm vessel produces a capillary pressure of only 3 m of water (corresponding to 0.3 bar or 0.03 megapascals). In trees that reach heights of over 100 m there will be a column of water 'hanging' in the stems and causing tensions of 9 bar or more at the top (100 m of water column or 10 bar, minus the counterbalancing atmospheric pressure). For this and other reasons, capillarity cannot cause the ascent of water in xylem conduits, although it is important in wall pores and in the pits of vessels which have much smaller dimensions.

A botanist, however, would probably cite a theory known as the cohesion theory of water transport¹ to explain the phenomenon. This theory has been around for more than a century, but in all that time no one has been able to supply unequivocal experimental evidence to prove it. Now at last supporting data are to hand, obtained independently by two groups and described by Holbrook *et al.* in *Science*² and by Pockman *et al.* on page 715 of this issue³.

E.D. Schulze/B. Stumpf

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REASONS

Hops, steps and jumps



EVER since 1771, when Luigi Galvani hung out frogs' legs in a thunderstorm, the determination of how muscle contraction takes place has been at the forefront of biophysical research. In two papers on pages 748 and 751 of this issue, Milligan, Sweeney and colleagues take one of the major steps in this quest. The power house of contraction is the protein myosin (red and blue in the figure) which acts against the filamentous protein actin (white in figure). Through sophisticated processing of electron microscopy images, the conformation of myosin with (red) and without (blue) bound ADP (a change thought to be a major part of myosin's powerstroke) is revealed. The head domain remains essentially immobile against the actin while an extended 'arm' pivots through upwards of 20° producing a potential movement of at least 35 Å. (The figure shows an actin filament decorated with smooth muscle myosin; graphics by A. J. Olson, D. Goodsell and G. Morris of Scripps Research Institute.)

Christopher Surridge

Unlike the cohesion mechanism, the root pressure mechanism requires metabolic energy to drive the uptake of solute and it does play a role in plants in the absence of transpiration. The highest root pressures measured are around 5 to 6 bar⁷. Because the osmotic pump mechanism cannot compete with the loss of water caused by transpiration, there is no positive pressure in the root xylem during transpiration. This would require large amounts of nutrient salts to be taken up at high energy cost and would cause problems in getting rid of the solutes in the shoot as the water evaporates.

Pressure probes⁸ can be used to puncture individual xylem conduits of transpiring plants and to measure xylem pressures directly⁹⁻¹¹, and the results suggest that pressures are much higher (less negative) than those obtained by conventional (indirect) techniques. Tensions were found to be hardly larger than 5 bar, with only subatmospheric (still positive) pressures in many cases. It was concluded that although there may be some contribution of tension to the overall flow, this is not the main mechanism. These conclusions have brought the validity of the cohesion theory into question.

Holbrook *et al.*² and Pockman *et al.*³ have now added to the debate. Both groups used a similar technique which circumvents the problems inherent in the more direct methods. Stem segments of trees were spun in a centrifuge to create

tensile forces and equivalent negative pressures acting on the xylem sap — a 40-year-old technique¹² adapted here for plants. Holbrook and co-workers detected changes in the water potential of the twig which were associated with the change in tension. Tensions of up to 15 bar could be created in the xylem by centrifugation and measured as an equivalent decrease in water potential.

Pockman *et al.* measured the increase in axial hydraulic resistance caused by cavitation as a function of tension (spinning rate). They were able to detect the critical tension that could be maintained in the xylem conduits. The results indicated that the vessels of the trees used remained water-filled at tensions of up to 35 bar, depending on the tree species — tensions that were never reached using the pressure probe. The authors then went a step further. They related the critical tension that caused cavitation to the external gas pressure that produced air seeding in stem sections, and found that these pressures were quite high and similar to those that caused cavitation during spinning. Although the approach is indirect, these cross-check experiments provide strong evidence for the maintenance of fairly negative pressures in xylem vessels of transpiring plants and support the cohesion-tension mechanism.

It might be argued that the range of tensions measured by Pockman *et al.* and by Holbrook *et al.* are too high, and that

liquid water is unlikely to sustain such tension without cavitation (although this can be avoided if gas nucleation is suppressed). Experimental values for the tensile strength of water range between 50 and 2,000 bar¹²⁻¹⁶. They are much lower than theoretical estimates in the absence of seeding, which has been put down to catalytic effects of the walls or of impurities present during the formation of gas nuclei¹⁷⁻¹⁹. Nevertheless, experimental values of the tensile strength of water, as measured in physics and physical chemistry, are sufficient to support the new results^{2,3}.

These results also serve as a warning that direct measurements of negative pressure with probes have to be taken with caution because of cavitation problems. We do not yet know whether current pressure probe techniques are able to measure tensions of the magnitude predicted by the indirect measurements, which may be as large as say 20 to 30 bar, without cavitation. So far, artificial systems have not been tested to demonstrate this crucial point, which means that conclusions drawn from pressure probe experiments about the existence of high tensions are suspect. We need further improvements in this technique.

Although the results of Pockman *et al.* and Holbrook *et al.* still provide only indirect verification of the cohesion theory, they indicate that it has a sound physical basis and that botanists have not been deluding themselves all these years after all. □

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All out for chromosome six

Leena Peltonen

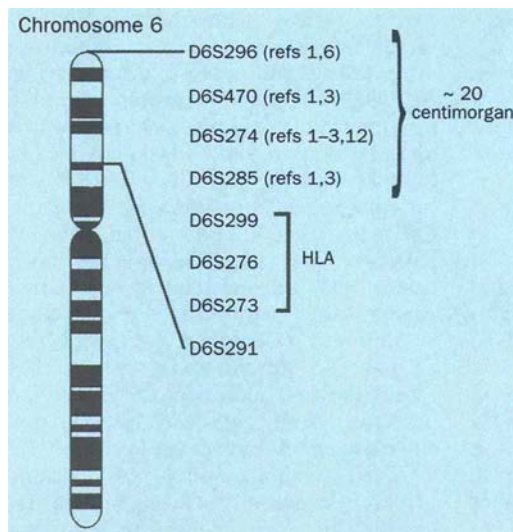
ANY news reporting genetic loci that predispose to schizophrenia is of great public interest, and is received with excitement by the scientific community. But over the past seven years the disappointments have been equally dramatic, in that such reports have usually been followed by others announcing failure to replicate the findings in independent patient or family studies. These turns of events have been frustrating but understandable, for two reasons — first, because diagnostic classifications of schizophrenia vary from study to study, making cross-comparisons difficult; second, because several genes (not to mention environmental influences) seem to be involved, so the condition is not amenable to the one-disease, one-gene approach that has successfully unravelled the molecular genetics of the thalassemias, cystic fibrosis and other monogenic disorders.

Three papers¹⁻³ and three pieces of correspondence⁴⁻⁶ in the November issue of *Nature Genetics* now bring a region on the small arm (p) of chromosome 6 to the centre of attention for schizophrenia researchers. Taken together, they describe results that at last provide firm evidence for one important locus that, when perturbed in some way, predisposes carriers to the disease. Although the chromosomal region is still very wide, potentially containing hundreds of genes, the excitement should not fade this time.

The first indication for the existence of a schizophrenia locus emerged in 1988, and was assigned to the long arm (q) of chromosome 5 (ref. 7), but this finding was never confirmed. When dense maps of polymorphic markers on individual chromosomes became available, systematic genome scans were initiated and the first promising findings were reported on chromosomes 22q and 6p. The schizophrenia locus on 22q was also implicated by an independent study based on a different strategy, namely a search for microdeletions on 22q in schizophrenia patients which turned up in two out of 100 cases⁸. But encouraging linkage data on chromosome 22 that emerged from two independent studies on large families in the United States unfortunately did not meet the strict criteria for statistical significance^{9,10}.

The data on chromosome 22q reflect the systematic problem in all linkage studies reported so far for potential schizophrenia loci. Because of the unknown mode of transmission of the disease and locus heterogeneity, and issues associated with the definition of gene frequency and ratio of phenocopies, multiple analytical models are required in statistical analy-

ses, which results in inflation of the lod scores (logarithm of the odds that two loci are linked closely enough to segregate together) obtained; lod scores are the statistical measure of the strength of the connection between locus and disease occurrence. The problem is amplified by the high number of markers tested overall, increasing the probability of an erroneous finding by chance. Using strict criteria for significance, such as those presented by Lander and Kruglyak¹¹ for example, the lod score in polygenic dis-



Region on the short arm of chromosome 6 associated with genetic susceptibility to schizophrenia, as discussed here. HLA indicates the region encoding human leukocyte antigens. Narrowing the search further for the particular gene or genes involved will not be straightforward: the process is much more complicated with polygenic disorders such as schizophrenia as opposed to cases where a disease is caused by mutations in one gene only. The majority of recombinations are not useful, because they may occur in individuals who are unaffected by the disease but carry the gene with incomplete penetrance. Also, restriction of the region by linkage disequilibrium would require a very dense marker map or an isolated population with only a few major ancestral mutations (ideally only one).

eases should be considerably higher than 3.0, which is the generally accepted threshold value for significance in monogenic diseases with a known inheritance pattern and a clear prior hypothesis for their molecular basis.

This problem of statistical significance also held for the first report of a susceptibility locus on 6p (ref. 12). The maximal lod score in this case, produced by extracting information from multiple loci and assuming locus heterogeneity, was 3.9, which in a genome-wide scan could still be obtained by chance one in 40 times. Consequently, independent studies were badly needed, not only to confirm the connec-

tion but also to provide more convincing statistical evidence.

So to the work now described in *Nature Genetics*. The genome-wide scanning for a schizophrenia locus described by Moises *et al.*² was carried out in three phases, a procedure that in principle has a built-in validation facility. In the first stage, only five family lines (pedigrees) with a total of 37 affected individuals from Iceland were used. Screening with 413 markers produced 26 'potential' loci, of which 10 were selected for the second stage and tested in a total of 65 families from numerous populations. This material revealed some evidence for linkage to four loci. When results from the first and second stages were combined and analysed, the statistical evidence for linkage increased slightly for loci on chromosomes 6p, 8p and 20. The most stringent significance level could be reached only for 6p, and then only after a third population sample was added — 113 schizophrenic patients and their unaffected parents from China.

The increasingly promising area on chromosome 6p was then approached directly by five other groups. Straub *et al.*¹ looked at an enlarged sample of the Irish material used in the original work¹² implicating 6p. In an analysis of an impressive 265 pedigrees, they have produced significant evidence for linkage on 6p. In an accompanying paper by Schwab *et al.*³, linkage analysis using affected sib pairs in 43 German families with a total of 78 sib pairs yielded encouraging lod scores with numerous markers spread over the crucial 30-centimorgan region. However, their data on their own do not meet the criteria that the connection is statistically sound. Likewise, the contribution by Antonarakis *et al.*⁶, which describes data from eight markers spanning the same region, tested in 57 pedigrees in the United States, provides only suggestive evidence.

Digestion of the data from all of these four reports^{1-3,6}, which take in a total of 430 families from different populations, and 113 schizophrenics with unaffected parents from China, provides evidence that chromosome 6p does indeed carry a locus that predisposes to schizophrenia; strong weight should be given to broad replication of the finding by so many different groups. Another unifying feature also emerges from the four studies: the observed locus heterogeneity. Although estimates of the fraction of each pedigree containing 6p-linked schizophrenics vary, 15–30% seems to be close to consensus. This would indicate a major predisposing

locus, especially taking into account the diversity of populations from which the families analysed originated.

The picture, however, remains far from clear. First, the diagnostic classification — the most disputed issue in psychiatric genetics — varies between the individual reports, creating confusion in the interpretation of the data. Moises *et al.*² used relatively strict criteria, scoring only individuals with schizophrenia or schizoaffective psychosis as affected, whereas Straub *et al.*¹ obtained the strongest evidence under a much broader diagnostic category — the significance for linkage drastically reduced with a more narrow disease definition. Schwab *et al.*³ and Antonarakis *et al.*⁶ also obtained the best statistical evidence using a narrow affected-status model. Besides, the mode of inheritance assumed is not uniform: Moises *et al.*, Straub *et al.* and Schwab *et al.* all obtained the best lod scores using a model in which the gene affected is expressed dominantly, as opposed to the recessive model of Antonarakis *et al.*

Finally, two pieces of correspondence, by Mowry *et al.*⁴ (reporting data on linkage studies in 45 families of African-American, Asian, Hispanic and Micronesian, as well as Caucasian, origin) and by Gurling *et al.*⁵ (on a total of 23 British and Icelandic pedigrees), reveal only negative or non-informative data on the critical region of chromosome 6p. This holds for both broad and narrow disease classifications, and for various inheritance models tested. Particularly for the families studied by Gurling *et al.*, it is impossible to find an explanation for this discrepancy either on the basis of diagnostic issues or the population concerned, when the data are compared to those revealing positive linkage. Chance effects might be involved, or some unidentified difference in diagnostic procedures.

How wide is the chromosomal DNA region concerned? Some suggestive lod scores were obtained on two regions some 20 centimorgans apart, probably reflecting different informativeness of markers in individual data sets and imprecise definition of inheritance pattern, rather than two true loci. Looking at the combined data, the wide region (close to 30 centimorgans long and containing maybe hundreds of genes) at 6p 21–24 remains the

best bet, but it will be no easy task to narrow the search further (see figure). Nonetheless, the large international effort now underway should yield dividends sooner or later, the prize being the identification of one of the genes that cripples the lives of millions of people and their families. Although it will be a long way from there to conceivable therapies, find-

ing the genes predisposing to schizophrenia is an essential first step in formulating new treatments for the disease. □

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DRUG ADDICTION

Cocaine abuse takes a shot

David W. Self

DRUG addiction can be viewed as a mental disease, one that is characterized by neurobiological disturbances and behavioural abnormalities such as compulsive drug taking and intense drug craving. Although there has been some success in treating heroin and alcohol addiction by pharmacological and behavioural means, there is currently no effective treatment for cocaine addiction. One possible strategy is to treat cocaine as an invading pathogen and to vaccinate the body against the drug, similar to vaccinating against other pathogenic diseases. Such an approach is described by Carrera *et al.* on page 727 of this issue¹, where they report the first successful demonstration of active immunization against the psychoactive effects of cocaine.

Active immunization against cocaine or heroin is accomplished by linking stable drug-like conjugates with a foreign carrier protein to stimulate the immune system to produce antibodies that subsequently recognize and bind the drug^{2,3}. When the antibodies bind the drug, they prevent it from diffusing across the blood-brain barrier, and thereby prevent the psychoactive effects. Using rats as their subjects, Carrera and colleagues show that pre-immunization against cocaine lowers drug concentrations in the brain and reduces the motor-stimulant effects following systemic injections of cocaine. Although not reported here, it would be of interest to determine whether this procedure also reduces cocaine's reinforcing properties, and thereby its addictive potential.

Cocaine's reinforcing properties result from its ability to prolong the synaptic action of the neurotransmitter dopamine by blocking its re-uptake into dopamine neurons⁴ in brain reward regions such as the nucleus accumbens⁵ and prefrontal cortex⁶. Accordingly, more conventional approaches to cocaine addiction include antagonists against dopamine or against cocaine, but these treatments have the respective drawbacks that they may exacerbate cocaine withdrawal symptoms⁷ or require continuous medication and patient compliance. Immunization-based

therapies therefore offer some advantages, in that they would lack psychoactive side effects and might involve only a few initial injections followed by intermittent booster shots (as, for instance, in immunizing against tetanus). In the present study¹, immunization against cocaine in rats produced increased levels of antibodies for at least 20 days following the final booster injection. However, a longer duration of months or years would be necessary for immunization to be effective in preventing or treating cocaine addiction in humans.

There are several other points to be addressed. First and foremost is whether the counteractive effect of cocaine antibodies can simply be overcome by increasing the self-administered dose of cocaine. Furthermore, daily exposure to cocaine may deplete levels of circulating antibodies through degradation of the antibody-cocaine complex, as found previously in a study of immunization against heroin². In work carried out *in vitro*, this problem was circumvented by using antibody-catalysed degradation of cocaine³. According to this rationale, the antibody acts as an enzyme, catalysing the hydrolysis of cocaine to inactive metabolites which then are released, allowing the antibody to bind another cocaine molecule and repeat the cycle. So catalytic antibodies may increase the capacity of immunization to block cocaine's psychoactive effects, even if higher doses of cocaine are self-administered repeatedly.

A potential hazard of immunization-based approaches to fight drug addiction is that some subjects may develop a hypersensitive allergic reaction to cocaine. Like reactions to bee stings, the allergic reaction to drugs could vary in intensity from mild swelling to full-blown anaphylactic shock resulting in death. Moreover, the possibility of an allergic reaction to drugs may be increased because relatively large doses, of cocaine in particular, are self-administered by abusers, and the route of administration (inhalation, for example) may concentrate the allergic reaction in a vital organ such as the lungs.

A final and central question is whether

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immunization-based procedures overlook the fundamental behavioural pathology of drug addiction, and hence may not effectively curb drug use. Specifically, although these and other procedures may block the reinforcing effects of drugs, they fail to target the core motivational and neurobiological features of drug addiction. Relapse to drug use in drug-dependent subjects is usually precipitated by episodes of intense drug craving that occur even after prolonged abstinence. A cocaine-dependent subject experiencing such intense cravings may simply choose to self-administer an alternative drug such as amphetamine. As shown in the study by Carrera *et al.*, immunization against cocaine does not block the psychomotor response to amphetamine. Conceivably, addicts could be immunized against a variety of drugs, but drug traffickers will undoubtedly synthesize new compounds with chemically unique structures, keeping one step ahead of our best medical efforts.

Perhaps a better strategy is to focus on the fundamental neurobiological mechanisms of drug craving and relapse. Persistent drug craving is probably caused by long-lasting neurobiological changes in brain reinforcement systems following chronic drug use⁸, and immunization-based procedures will be ineffective at reversing or restoring these systems to normal. Current pharmacotherapeutic approaches, aimed at preventing relapse by reducing drug craving through reversal of the drug-induced changes in brain function, may offer a promising alternative for treating drug addicts. In contrast, immunization may have greater benefit in preventing drug addiction, or in detoxifying drug overdose patients, rather than in treating drug addiction itself.

In this sense the work described by Carrera *et al.* is both innovative and potentially useful in certain circumstances. But drug addiction is a complex mental disease and, as with other mental diseases, there are no quick fixes. □

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Blueprint for success

Olivier Kahn

ONE of the first synthetic coordination compounds ever to be reported (in 1710) was Prussian blue, a pigment obtained by the reaction of the diamagnetic $[\text{Fe}(\text{CN})_6]^{4-}$ complex anion with the Fe^{3+} cation¹. Since then, quite a few compounds belonging to the same family have been prepared, many of them showing unusual physical properties. The latest striking example is given on page 701 of this issue, where Verdager and co-workers report² that a novel Prussian-blue-like phase behaves as a magnet below a critical temperature of 320 K.

Molecular magnetism is a new field of research which has emerged over the past decade or so³. The heart of the field concerns the design and synthesis of molecular assemblies that show bulk properties such as long-range magnetic ordering or bistability with hysteresis.

Before looking in more detail at the magnetic properties reported by Verdager and co-workers, let us briefly sum up what we already know from studies on Prussian-blue-like phases. Prussian blue itself owes its name to its colour: mixing together a pale yellow aqueous solution of $\text{K}_4[\text{Fe}(\text{CN})_6]$ with a light orange solution of an $\text{Fe}(\text{III})$ salt immediately results in a deep blue precipitate of $\text{Fe}^{\text{III}}_4[\text{Fe}(\text{CN})_6]_3 \cdot 15\text{H}_2\text{O}$. This compound presents an intense absorption band at 700 nm, due to a transition from the ground state to an excited state in which an electron is transferred from an $\text{Fe}(\text{II})$ to an $\text{Fe}(\text{III})$ site (ground $\text{Fe}(\text{III})_A\text{Fe}(\text{II})_B$

to excited $\text{Fe}(\text{II})_A\text{Fe}(\text{III})_B$). Prussian blue may be considered as the archetype of mixed valence compounds, containing two identical metals in different oxidation states. These compounds have played, and continue to play, a crucial role in the study of electron transfer phenomena⁴.

Prussian blue's attraction for chemists and physicists lies not only in its optical properties but also in its magnetic properties: it shows a long-range ferromagnetic ordering at $T_c = 5.6$ K (refs 5, 6). Of course, this critical temperature is low, but only the $\text{Fe}(\text{III})$ sites carry a spin, whereas the $\text{Fe}(\text{II})$ sites are diamagnetic. It follows that the magnetic interactions must occur between next-nearest ions through the 10.6-Å-long $\text{Fe}(\text{III})\text{--C--N--Fe}(\text{II})\text{--N--C--Fe}(\text{III})$ linkages. The possibility of propagating magnetic interactions through extended bridging networks between widely separated spin carriers seems to be specific to molecular compounds; it is due to the strong spin delocalization from the metal ion towards its nearest neighbours. The presence of strong spin densities on the nitrogen and carbon atoms of the cyano groups has been experimentally observed by polarized neutron diffraction⁷, a technique that allows spin-density maps to be plotted.

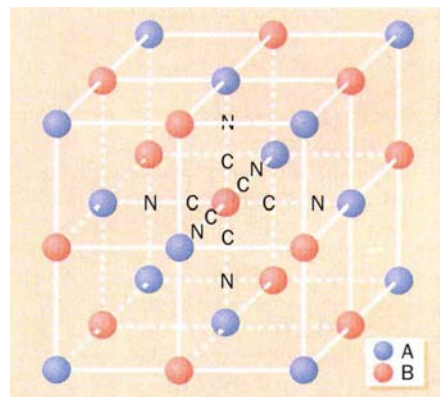
Magnetic ordering temperatures that are much higher than that found for $\text{Fe}^{\text{III}}_4[\text{Fe}(\text{CN})_6]_3 \cdot 15\text{H}_2\text{O}$ could be anticipated for Prussian-blue-like phases containing two magnetic metal ions (for

Ordering in 'Prussian-blue' phases

In Prussian-blue-like phases of general formula $\text{A}_k[\text{B}(\text{CN})_6]_l \cdot n\text{H}_2\text{O}$, the metal sites A and B and the lattice as a whole are highly symmetrical. Both A and B are in octahedral surroundings, where the 3d valence orbitals are split into a low-lying (t_{2g}) and a high-lying (e_g) subset. For B only the low-lying t_{2g} orbitals are occupied, so the magnetism shown by the compound overall depends on A.

If all the unpaired electrons of A occupy t_{2g} orbitals, the A–B interaction will be antiferromagnetic, and the compound will be a ferrimagnet, provided that there is no compensation of the spin moments. If the unpaired electrons of A occupy some t_{2g} and some e_g orbitals, the A–B interaction will again be antiferromagnetic, but less pronounced than in the previous case, and the compound will be a ferrimagnet with a lower critical temperature. Finally, if the unpaired electrons of A only occupy e_g orbitals, the A–B interaction will be ferromagnetic, and the compound will be a ferromagnet.

O. K.



Idealized structure of bimetallic Prussian-blue-like phases.

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instance, chromium and nickel), and two groups led by Verdaguer⁸ and Girolami⁹, respectively, are currently involved in a systematic investigation of these compounds. The general formula of the bimetallic Prussian-blue-like phases is $A_k[B(CN)_6]_l \cdot nH_2O$ where A is a high-spin and B a low-spin ion. (The ground state of a transition metal ion results from a competition between Hund's rule, which favours the high-spin state, and electron pairing, which favours the low-spin state. Usually, Hund's rule takes over and the ion has a high spin. In the hexacyanometallates, on the other hand, the electron pairing energy is exceptionally strong, and the ion has a low spin.) For $k = l$, the basic (idealized) structure is face-centred cubic with A–N–C–B linear linkages along three perpendicular directions, as shown in the box.

These compounds may be three-dimensional ferromagnets, antiferromagnets or ferrimagnets, depending on the nature of the A–B interaction. In ferromagnets, the two spin sublattices couple so that all spins are parallel. For both antiferro- and ferrimagnets, the two spin sublattices A and B couple in an antiparallel way. Antiferromagnetism corresponds to strict compensation of these sublattices, and ferrimagnetism to incomplete compensation so that there is a non-zero resulting spin. One of the very appealing aspects of the magnetic Prussian-blue-like phases is that these materials are excellent candidates on which to test theoretical models describing the magnetic interaction, in particular as far as symmetry rules are concerned.

In theory³, if the unpaired electrons from A occupy a set of singly occupied orbitals a_μ , and those from B occupy orbitals b_ν , the overall interaction between A and B arises from a sum of contributions involving pairs of these orbitals. When a_μ and b_ν have the same symmetry, the contribution is antiferromagnetic; configurations in which neighbouring spins are alternately up and down are favoured. When the orbitals have different symmetry, the contribution is ferromagnetic; it favours the up-up spin structure. Finally, when there are both antiferro- and ferromagnetic contributions, the former dominate, and the overall interaction is antiferromagnetic. Just how this applies to the Prussian-blue-like phases, and how it affects their critical temperatures, can be seen in the accompanying box.

So far, all known examples of Prussian-blue-like phases strictly obey these predictions. In the particular case of the compound reported by Verdaguer and co-workers², containing V(II), V(III) and Cr(III) sites for which all the magnetic orbitals have the same symmetry, the V(II)–Cr(III) and V(III)–Cr(III) interactions are strongly antiferromagnetic, and

the compound behaves as a ferrimagnet with a high critical temperature.

Of course, a magnet is not characterized just by its critical temperature; its saturation magnetization and its coercivity are also important. In particular, it is the coercivity defined by the width of the magnetic hysteresis loop that confers a memory effect on ferri- or ferromagnetic materials. At least 100 Oe would be needed for $V[Cr(CN)_6]_{0.86} \cdot 2.8H_2O$ to be useful in recording media. In the present case, the saturation magnetization is limited to 0.15 Bohr magnetons, owing to the weak value of the resulting spin per repeat unit. The coercivity is also weak; at 10 K, the value of the coercive field is only 10 Oe. Perhaps the use of more anisotropic spin carriers, which would prevent the magnetic domains from rotating freely as the field is applied, would result in an increased coercivity.

Strictly speaking, the phase that has been synthesized by Verdaguer and co-workers, $V[Cr(CN)_6]_{0.86} \cdot 2.8H_2O$, is not a molecular compound, but rather an amorphous and non-stoichiometric extended solid. However, this room-temperature magnet is prepared from the molecular precursor $[Cr(CN)_6]^{3-}$, and the synthesis of such a material may be considered as a cornerstone in the field of molecular magnetism. This is not the first room-temperature magnet to be synthesized from molecular precursors — a few years ago, there was a report¹⁰ of a compound of that kind, of formula $V(TCNE)_2$ — but the new compound is in many respects better characterized. Remarkably, it was obtained in a rational way, rather than as the result of serendipity, and all its magnetic characteristics (T_c , saturation magnetization, coercivity) are in line with the theoretical models developed in molecular magnetism. $V[Cr(CN)_6]_{0.86} \cdot 2.8H_2O$ is an interesting material in its own right, and an excellent example on which to learn (or to teach) the basic concepts of molecular magnetism. □

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Animal vegetables

ANIMAL rights campaigners complain ever more vociferously about the cruelties of modern agribusiness. They have a point. Hens in cages, pigs in sweatboxes and calves in crates can be raised very cheaply, but their lives are miserably restricted. There must be a better way.

Daedalus once proposed to put such creatures under permanent anaesthesia. This would pose many tricky problems, and might contaminate their meat. He now reflects that consciousness is the highest and most volatile of brain functions anyway. It can be abolished not only by drugs, but by oxygen deprivation or even by simple impact. The effect is usually brief, but can be permanent. A head injury sometimes leaves its victim alive but in an irreversible coma. His conscious brain centres are destroyed, while the lower centres that control his heart and lungs are untouched.

This tragedy for a man, says Daedalus, could be merciful release for a farm animal. An operation like prefrontal lobotomy but a bit further back, or partial asphyxiation with nitrogen, or even a well-judged bang on the head, could end the conscious life of a sheep or cow. It would be reduced to a comatose 'animal vegetable'. Unable to eat or drink, it would die in a few days. By itself, this simple treatment would be ideal for animals destined for slaughter in a distant country. They could be transported as passive animal vegetables, avoiding the usual miseries of such a long and comfortless journey.

An animal vegetable might, however, be kept alive indefinitely. A hen, for example, has many vital reflexes controlled by its spinal cord. It can even run after its head has been cut off. A well-designed brain operation could put it into a permanent coma, while leaving untouched its reflex ability to eat (or at least to swallow grain or drink water placed in its beak) and to lay eggs. Such hens could pass their whole battery lives in merciful oblivion. Passive and inert, they would not attack each other and would need no caging: the battery could be simpler and more profitable.

The same principle should apply to sweatbox pigs, crated calves and geese for *pâté de fois gras*. Once the proper neurosurgery had been worked out, all these creatures could be swiftly released from their miseries, leaving their breathing bodies to grow and develop for human benefit. Packed in racks, their food supplied and their wastes removed by machinery, the zomboid creatures would lift agribusiness to its ultimate peak of efficiency. And the industry could boldly claim that none of its charges suffer in the least.

David Jones

Whose baby are you?

SIR — It is almost inevitable for parents to be told that their babies look just like them, but the people who suggest this to the pleased parents have the advantage of already knowing that these are their children. Without this advantage, can people pick the correct progenitors from sets of possible parents? Our experiments provide two answers: children in general do not look enough like their parents for a resemblance to be detected, with the one exception that one-year-olds look like their fathers.

Subjects were shown pictures of offspring, and rated the similarity between each of them and three possible mothers or fathers, one of whom was the actual parent (Fig. 1). Subjects performed this task for both male and female children at ages one, ten and twenty years. They also rated the similarity between children at these three ages, and between pictures of parents taken when their children were one and twenty.

The subjects were very accurate at



FIG. 1 Twenty-four Caucasian families provided a photograph of a child at age one year, a photograph of the mother and one of the father, both taken when they were one year old. Twelve of the children are now adults, allowing us additionally to gather photographs of the child at 10 and 20 and a recent photograph of the parents. Twelve sets with boys and twelve with girls were obtained. Subjects rated the resemblance between the three ages of offspring and sets of possible parents, between the children at the three ages, and between the parents at different ages. They compared each stimulus picture with three possible matches, one of which was a true match. They rated the resemblance between the stimuli and each of the possible matches on a 0–10 scale of increasing resemblance. In all, 122 subjects participated, and each possible match was rated by 18–21 of these raters. The order of the stimulus pictures, the three possible matches, and the three child ages were counterbalanced. The figure shows an example of a one-year-old and three possible fathers. The correct match is b.

detecting the similarities between the pictures of the parents taken 20 years apart (Fig. 2a). The children were also recognizable from one age to another, indicating that the pictures were of high enough quality for their individual characteristics to be visible. Males and females were equally recognizable over time.

The 20- and 10-year-old children were no more similar to their actual mothers and fathers than to random parents (Fig. 2b). One-year-olds could not be matched to their mothers. In fact, the only reliable similarity was between one-year-olds (both girls and boys) and their fathers.

Children in general do not look enough like their parents to allow judges who do not already know of the relationship to spot the resemblance. Assertions that children look just like their parents could be due to a desire to please the parents, picking just the features that randomly show some resemblance, or commenting on only the offspring who happen to be similar. Alternatively, resemblance could depend on characteristics that still pictures cannot capture.

There may be an evolutionary rationale for the unique resemblance between one-year-olds and their fathers. While a mother can be quite sure that the baby is hers, no matter what it looks like, the father cannot. It could then be to a baby's advantage to look like the father, to encourage paternal investment. Daly and Wilson¹ argue that people are biased to say there is a resemblance between children and fathers to reassure the dads. They collected evidence that mothers, fathers, relatives and friends think babies look more like their father than their mother and suggest that these subjective reports are due to an evolutionarily advantageous bias, never considering that they may be accurate. (It is also the case that the evolutionary pressure should be to make the baby look more like the father than other men, not more like the father than the mother. Babies who simply look masculine are no reassurance to nervous fathers.) Finegan and Mackenzie² have also reported that babies look more like their fathers than their mothers, although not more like unrelated men than unrelated women.

Because both male and female babies can be matched to the father, it is not simple sex-linked inheritance. One possibility is that

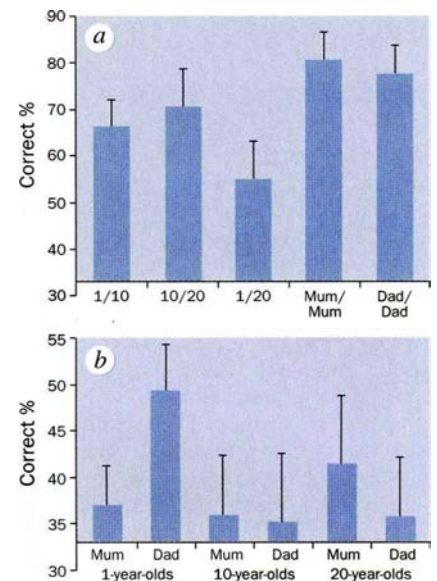


FIG. 2 The percentage of subjects who gave the correct match the highest rating was computed for each stimulus, and these scores were compared with the chance level of 33.3%. a, The raters could match: one-year-olds to themselves at 10 (66.3%, $t(11) = 5.64$, $P < 0.0005$), 10-year-olds to themselves at 20 (70.6%, $t(11) = 4.62$, $P < 0.001$), one-year-olds to themselves at 20 (55.0%, $t(11) = 2.62$, $P < 0.05$), mothers to themselves at ages separated by 19 yr (80.6%, $t(11) = 8.0$, $P < 0.0001$), and fathers to themselves (77.6%, $t(11) = 7.2$, $P < 0.0001$). b, The only significant familial resemblance was reported between one-year-olds and their fathers (49.2%, $t(23) = 3.21$, $P < 0.005$). This ability to pick out the biological fathers was true for both baby boys (50.5%) and baby girls (48.0%), and was essentially as good as the ability to match these infants to themselves at 20.

the variability of babies' faces is more similar to the variability of fathers' faces than of mothers'. Features that distinguish women from each other, such as cheekbone prominence, may not help with baby identification, because they are obscured on their faces, while the features that are salient on men might be visible on one-year-olds. Another possibility is that the facial morphology of a baby is more determined by the father than by the mother. Differential maternal and paternal gene expression has been documented, with DNA methylation possibly playing a role in such 'genomic imprinting'.³ It may be to a mother's advantage for the expression of her genes to be limited in determining the appearance of a child if it causes the father to recognize the infant and care for it.

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Lifting the Taung child

SIR — The Taung child, *Australopithecus africanus*¹, is a key fossil to our understanding of hominid evolution. It is, however, accompanied by an exceptional fossil fauna comprised of mainly relatively small animals. This has led to much analysis and speculation about the possible agent responsible for the accumulation of this fauna². Large carnivores or even the ape-man himself have been proposed as the most likely candidates. Recently, Berger and Clarke³ suggested that the primary collecting agent of the Taung child and the associated fauna was a large bird of prey. Support for this hypothesis was obtained by comparison of the Taung fauna with that found below nests of large African eagles. The most likely candidate species is the crowned eagle *Stephanoaetus coronatus*³, which was anecdotally reported to have attacked, and nearly killed, a 7-year-old child of approximately 20 kg. The body mass of the Taung child was probably 10–12 kg (ref. 3).

I used biomechanical information^{4,5} about bird load-lifting capacity to test whether a raptor the size of a crowned eagle would be capable of carrying a prey the weight of the Taung hominid. During a short anaerobic sprint exertion, the load-lifting capacity of a crowned eagle is approximately 6.1 kg (ref. 4; assuming a 4.12-kg eagle body mass⁶), well below the body mass of the Taung child. In sustained flapping flight, the fuel load-carrying capacity of an eagle is only about 1.7 kg (ref. 5). However, because fat used as fuel is uniformly distributed around the body, drag is minimized compared with a load of prey held in the talons. Therefore, this represents an upper limit to the sustainable load-carrying capacity. The distance between the hominid savannah habitat and the deposition site was probably substantial³, and so an eagle could have carried little more than the skull over this distance.

Biomechanics thus show that if a large bird of prey collected the Taung fauna, the Taung child itself must have been dismembered before being brought to the eagle's nest.

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Energetic motion detection

SIR — Motion detection has been widely investigated using random-dot kinematograms (RDKs). Under appropriate conditions, motion may be seen between a random-dot image and its displaced counterpart¹. Here we attempt to discriminate between the two classes of model that can account for this.

Proponents of energy-based models argue that low-level motion sensors detect motion energy at a range of spatial scales². The directional outputs of the detectors are later combined to indicate the overall direction of movement of an object. Alternatively, edge-based models suggest that the edges in an image are located and then motion is detected at a single scale by forming correspondences between displaced edges³. Both models can account for the observation that the maximum displacement for motion detection ($d_{\max}$) increases when a RDK is low-pass filtered. For energy-based models, this is because low-spatial-frequency detectors have larger receptive fields and tolerate larger spatial displacements. For edge-based models the increase is because low-pass filtering increases the separation between edges.

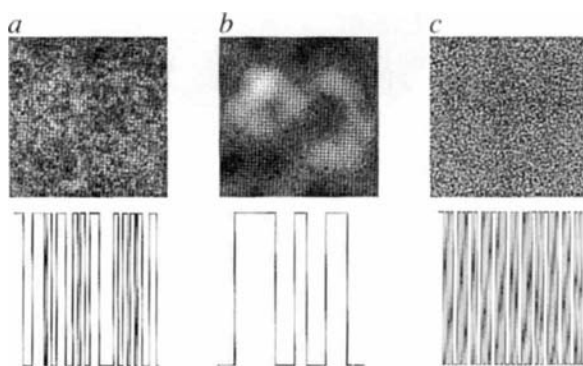
$d_{\max}$ is surprisingly small for white noise, given the presence of low spatial frequencies¹, while the absence of an effect on $d_{\max}$ of initial low-pass filtering is unexpected⁴. Proponents of energy-based models must resort to special pleading to explain these effects (that is, that high spatial frequencies mask low⁴). In edge-based models, direction discrimination is based on the edges in the pattern after initial blurring by the visual system. The low-pass filtering of the stimulus must exceed that of the visual system before

there is any noticeable effect. A cornerstone of the argument supporting edge-based models is the recent finding that observers could not see motion between a binary noise image and some of its low-pass-filtered counterparts⁵. In these images, there is little correspondence between the location of the edges in the two frames, so edge-based models predict that motion would not be detected. There is motion energy at common (low) spatial frequencies, however, so energy-based models predict that motion would be seen.

White-noise patterns in RDKs generally contain equal energy at all spatial frequencies. It has been suggested that the spatial characteristics of cortical cells are matched to the spectra of natural images, thus providing an efficient representation of the natural environment^{6,7}. Both simple and complex cortical cells have frequency bandwidths that are approximately constant in octaves^{8,9}. Thus, white-noise patterns produce activity skewed towards high spatial frequencies because the frequency bandwidth of cortical cells increases with peak frequency; cells tuned to high spatial frequencies respond the most. To provide a more appropriate test, noise patterns were filtered to the $1/f$ magnitude spectrum characteristic of natural images, where magnitude scales inversely with spatial frequency. The response of visual cortex cells to $1/f$ patterns is therefore equal across spatial scales and the neural representation is broad and flat.

We measured $d_{\max}$ using $1/f$ noise patterns which were subsequently low-pass filtered or high-pass filtered (Fig. 1, top row). The observer reported the direction of displacement in each trial. Direction

FIG. 1 The top row illustrates stimuli used. *a*, An 8-bit (gaussian) white-noise field, filtered to have a $1/f$ relationship between spatial frequency and amplitude; *b*, low-pass-filtered version of *a*; *c*, high-pass-filtered version of *a*. Contrast has been normalized for illustrative purposes, but in the experiment contrast was not normalized. The second row shows the edges in a one-dimensional section through each noise sample after low-pass filtering to simulate visual blurring. The edges were located at the zero crossings in the second spatial derivative of the blurred luminance profiles¹². There is little correlation between the edges in each pattern, and any edge-matching algorithm would be unable to identify correct matches for motion detection between filtered and unfiltered $1/f$ patterns. It may be possible to match the peaks between the $1/f$ and the low-pass image, but it would not be possible to match them between the $1/f$ and the high-pass image. Four main conditions were examined: both images were filtered in the same cut-off (low- or high-pass); one image was $1/f$ noise and the other was filtered (low- or high-pass). The order in which the images were presented was examined separately (full experimental details are available on request). Direction discrimination was possible between broad-band $1/f$ noise and filtered $1/f$ noise, despite the absence of correlation between the edges in these images.



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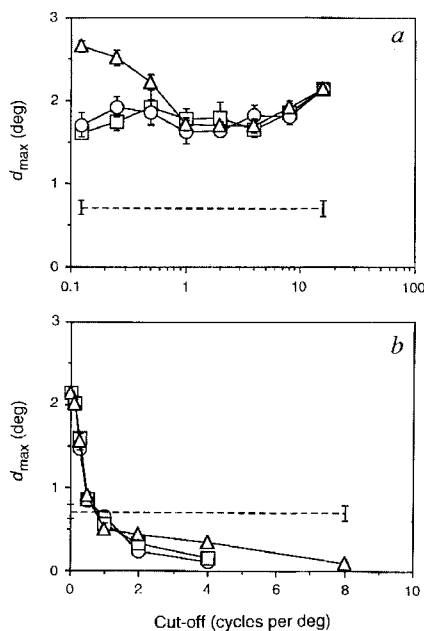


FIG. 2 $d_{\max}$ as a function of the cut-off frequency of filtered $1/f$ noise patterns. $d_{\max}$ corresponds to the displacement at which there were 20% errors in direction discrimination. The data are shown for a naive observer (similar data are available on request for a second observer). a, Low-pass data; b, high-pass data. The cut-off of the filter is shown on the x-axis in cycles per degree. For the low-pass condition, 16 c deg^{-1} represents the unfiltered $1/f$ noise pattern (the Nyquist limit of the image). For the high-pass condition, 0 c deg^{-1} represents the unfiltered $1/f$ noise pattern. Graphs show estimates of $d_{\max}$ when: both images were filtered (Δ); when the first image was broad-band ($1/f$) and the second was filtered (∇); and when the first image was filtered and the second was $1/f$ ($\circ$). The broken line shows the estimate of $d_{\max}$ for the unfiltered 8-bit noise. Error bars show $\pm 1 \text{ s.e.}$; $d_{\max}$ was relatively unaffected by low-pass filtering except when the cut-off was very low, where there was a slight increase in the mixed image sequence. When either image was high-pass filtered, $d_{\max}$ scaled with the lowest spatial frequencies in the high-pass image. Observers could not see motion between unfiltered $1/f$ noise and high-pass patterns with the highest cut-off, which may be related to the low contrast of the high-pass-filtered image.

discrimination was measured for displacements between two images which were either both filtered or only one of which was filtered (the other was $1/f$). When one of the images was filtered and the other was broad-band, direction discrimination was possible under almost all conditions (Fig. 2). In these images, there was little or no correlation between the edges in each image (Fig. 1, bottom row), but there was motion energy at common spatial scales. Furthermore, motion may be seen between unfiltered $1/f$ and band-pass-filtered $1/f$ patterns of various central frequencies, suggesting that the human motion system has equal access to information across spatial scales for natural images¹⁰.

Although direction discrimination may,

in principle, be performed by a high-level feature-tracking mechanism¹¹, this cannot explain motion detection between unfiltered and filtered images. These results show that low-level motion detectors are energy-based and reconcile the different explanations of the lack of effect on $d_{\max}$ of initial low-pass filtering. Such results are expected when the response bias to high spatial frequencies in white noise patterns is considered. The neural representation of white noise is effectively high-pass-filtered. It is therefore unsurprising that high spatial frequencies are dominant and that motion may not be seen between white noise patterns and their low-pass-filtered counterparts. Although there is undoubtedly initial low-pass filtering due to the limited resolution of the visual system, the separation between edges in the neural representation of an image cannot account for the ability to integrate motion between images whose edges are uncorrelated.

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MORGAN REPLIES — Mather and I⁵ showed that motion detection is possible between pairs of random noise patterns, one of which has been low-pass-filtered (blurred), even though the appearance of the blurred and unblurred patterns is distinctly different. This was explained by a motion-detection model in which the patterns are matched following a visual filtering stage in which high-spatial-frequency components are substantially attenuated³. After filtering, the initially blurred and initially unblurred patterns have a spatial structure that matches sufficiently closely for motion to be detected. Only when the difference in blur is extreme does the matching process break down, and detection by human observers fail⁵.

Bex *et al.* have now shown that correct matching can also be achieved between filtered and unfiltered versions of a $1/f$ -scaled gaussian noise pattern. Scaling by $1/f$ selectively attenuates high spatial frequencies, and so would be expected to reduce the effects of subsequent low-pass filtering, as Bex *et al.* find. Their result

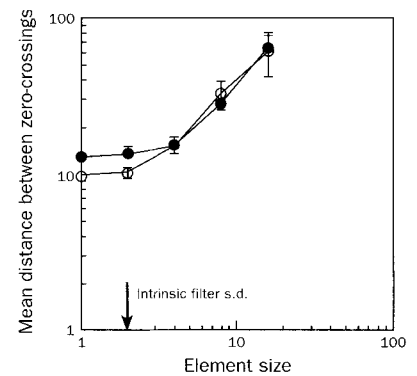


FIG. 3 The zero-crossing structure of random binary noise viewed through a filter that attenuates high spatial frequencies is little affected by $1/f$ filtering. The noise consisted of elements that were white or black with equal probability and which had the size shown on the abscissa. (Similar results are obtained with gaussian noise as used by Bex *et al.*) The noise pattern was convolved with a laplacian-of-gaussian filter with a standard deviation of 2 units, and the mean distance between zero-crossings was measured (ordinate). $\circ$, Unscaled; $\bullet$, $1/f$ -scaled. There were 10 independent simulations for each data point and the vertical bars represent standard deviations. For further details of the model see ref. 3.

would be expected if the spatial structure of the filtered and unfiltered patterns were sufficiently correlated following further filtering by an intrinsic visual filter. A simple statistical description of the edge structure of a noise pattern is provided by the mean distance between zero-crossings in the second spatial derivative¹³. This statistic is substantially unaffected by $1/f$ filtering (Fig. 3), except that, at small element sizes, the interval is about twice as great for $1/f$ -filtered patterns, in agreement with Bex *et al.*'s finding that the $1/f$ patterns have two to three times larger $d_{\max}$ values than pure noise. We predict that this difference would disappear with larger element sizes. The value of $d_{\max}$ for pairs of white noise should not be increased by $1/f$ filtering of one of the pair. If either of these two predictions is wrong, our model could be rejected.

The opposition that Bex *et al.* propose between 'motion energy' and 'edge matching' models of motion detection does not necessarily reflect different mechanisms. The zero-crossing pattern is a convenient description of its statistical structure, which is basic for understanding its effects, whether on a motion-energy detector or otherwise. In describing the

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zero-crossing structure of the stimuli, we do not mean to imply that zero-crossings are explicitly represented. Other descriptions of the pattern structure are possible (including the centroids of zero-bounded regions⁵), and we have no doubt that the positions of peaks of local energy¹⁴ would give a similar picture. The critical question is whether $d_{\max}$ is determined by the statistical structure of the pattern, and if

so, at what spatial scale or scales. Bex *et al.* do not provide a quantitative model of $d_{\max}$ based on 'motion energy', and until one is available it would be premature to reject an existing model that has made some successful predictions.

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Mariner transposons in humans

SIR — The *mariner-Tc1* gene superfamily represents a diverse collection of DNA-based transposable elements with broad phylogenetic distribution¹. We have now identified two *mariner* transposons in the human genome which share both structural and sequence similarity to elements previously described in arthropods, nematodes and planaria¹⁻³. The two human elements are members of divergent *mariner* subfamilies, suggesting multiple instances of horizontal transfer of these elements into the human genome.

Mariner elements, first described in *Drosophila*⁴, consist of a transposase gene flanked by two short terminal inverted repeats. This structure is similar to a variety of other DNA-based transposable elements⁵, including the *Ac* element in maize. The autonomous *Ac* elements are associated with a family of non-autonomous *Ds* elements⁶. Whereas *Ac* and *Ds* elements are defined by the same 11-base-pair (bp) inverted repeats, the *Ds* elements vary considerably in both size and internal sequence. Small, divergent, non-autonomous elements that share sequence similarity with the autonomous forms only within the terminal inverted repeats are a commonly observed feature in DNA-based transposons⁵.

The human *mariner* transposons were detected by identifying two families of putative non-autonomous transposons in

the genome. These elements were identified as short, conserved, inverted repeated domains present in multiple loci⁷. The members of the first of these families are defined by a 45-bp inverted repeated domain designated *hum1* (*a* in the figure). Small putative transposable elements can be easily identified in a variety of human genes by screening the available databases with the consensus *hum1* repeat⁸. Two oligonucleotide primers were designed using this repeat, and used to direct two rounds of polymerase chain reaction (PCR) amplification with human genomic DNA as a template. A 1.4-kilobase amplification product from these reactions was sequenced and found to be a *mariner* transposable element, designated *humar1* (GenBank locus HSU38613; *b* in the figure). The same element has also been cloned from a human genomic library using *humar1* PCR product sequences as probes.

The structure of *humar1* is similar to previously described *mariner* elements. As shown in the figure (*b*), the *hum1* repeats defining the element flank smaller (29 bp) imperfect inverted repeated domains. We have sequenced two additional genomic clones containing *humar1* elements (GenBank loci HSU38614 and HSU38615) which are defined only by the internal 29-bp repeats and lack the *hum1* domains. This suggests that the internal repeats represent the authentic terminal

repeats of this transposon. The transposase pseudogene is interrupted by four frameshifts and numerous stop codons. The deduced amino-acid sequence of the transposase is similar to that of the *mariner* transposase from lacewing (*Chrysoperla plorabunda*; PIR locus S36925), indicating that this element is a member of the irritans subfamily of *mariner* elements⁹.

We have identified a second human *mariner* element, a member of a second subfamily. Similar to *humar1*, this element was found by first identifying a second family of small, putative, non-autonomous transposons defined by inverted repeated domains (designated *hum7*, consensus inverted repeated domain sequence: TGGTGCAAAAGTAATGCAGTTT-TGCCA)*. When this sequence was used as a database query⁸, one of the returned sequences (GenBank locus HUMTCRB, positions 495,300–497,513) was found to contain a *mariner* element defined by two *hum7* repeats. The DNA sequence of the transposase pseudogene at this locus is interrupted by four frameshifts, numerous stop codons and a 950-bp insertion. The deduced amino-acid sequence of the putative transposase is similar to that of a planarian (*Dugesia tigrina*) *mariner* transposase (GenBank locus DTPM9), suggesting that this element is derived from the cecropia subfamily of *mariner* elements⁹.

The two *mariner* elements described here represent two divergent subfamilies, consistent with multiple horizontal transfer events involving *mariner* transposons¹. We have not yet identified intact (mobile) human *mariner* elements, which would represent potential sources of transformation vectors.

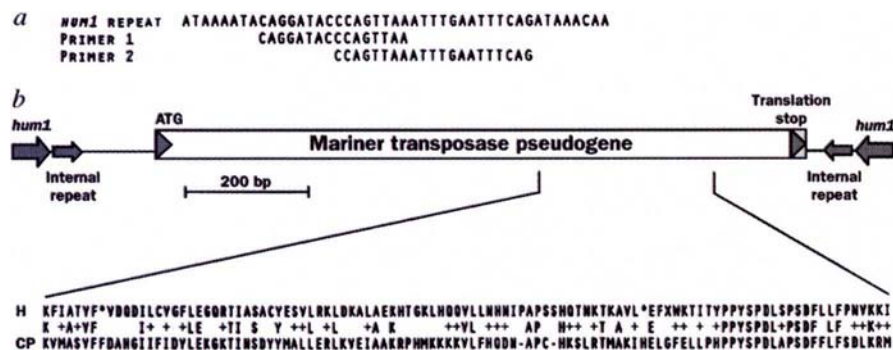
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Sequence of the *hum1* repeat and structure of the *humar1* transposon. *a*, Consensus sequence of the *hum1* inverted repeated domain. The oligonucleotide PCR primers used to amplify the *humar1* transposable element are indicated. *b*, Structure and partial transposase deduced amino-acid sequence of the *humar1* element. The relative positions of the *hum1* and internal 29-bp inverted repeats are indicated by arrows. A portion of the carboxy-terminal domain of the transposase was translated (asterisks indicate stop codons) and submitted to the BLAST Network Service of the National Center for Biotechnology Information⁸. The sequence alignment of the *humar1* (H) and *C. plorabunda* (CP) transposase was returned. Positions of identity are shown; + indicates a conservative change.

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*During the preparation of this manuscript, we became aware that the *humar7* element described here was also identified by Hugh Robertson, University of Illinois, using *mariner*-transposase-specific sequences (H. M. Robertson, personal communication), and that partial *mariner* transposase sequences have been reported¹⁰.

What price philosophy?

Charles Tanford and Jacqueline Reynolds

Ludwig Boltzmann: His Later Life and Philosophy, 1900–1906. Edited by John Blackmore. Kluwer: 1995. Pp. 266. Dfl140, \$89.50, £59.

WHEN we were students, Ludwig Boltzmann was our hero, more so than, say, Einstein, because we were chemists and Boltzmann is more immediately relevant to chemistry. His distribution law and the eloquent equation $S = k \log W$ were essential for us. How could one understand chemistry without them? And they retain their lustre to this day — Boltzmann's equations remain indispensable, on subjects extending from interpretation of thermodynamic or kinetic data for biological processes to current debates about black holes and other cosmological matters.

There is also of course a well-known dark side to Boltzmann's life — his gradual abandonment of physical science for philosophy and his death by suicide, hanging himself (at the age of 62) while on a family holiday at the seaside. John Blackmore's documentary history is confined to this period, to the last years of Boltzmann's life. He has assembled a collection of documents, letters to and from and about Boltzmann, interspersed with lecture notes and anecdotal recollections, arranged in an admirable way, so that the ageing man himself emerges with rare clarity from the printed pages. (Poorly printed, we might add, for rarely have we seen a book with more gross typographical errors.) But the reader should be warned that there is nothing here about Boltzmann in his prime. For example, 17 letters between Boltzmann and Lorentz dating from 1886 to 1897 are mentioned as existing but there is no hint of their content.

As the book's title implies, there are two ways to peruse this book. One can focus on philosophy and try to assess Boltzmann's role in the field — for example, did Boltzmann influence Wittgenstein? Or one can focus on the life itself. We (the reviewers) are hardly competent for the former, and the latter is probably in any case more congenial to most readers of *Nature*. But, as it happens, the central theme in either approach must be the same, the long battle — an intimate part of scientific folklore — between 'energeticists' and 'atomists', personified by Ernst Mach and by Boltzmann respectively. The energeticists harked back to Bishop Berkeley and his denial of anything not directly evident to the senses, which in Mach's time included atoms and

molecules. The atomists, of course, were the heirs of Newton's corpuscular mechanics. In Germany and Austria (unlike the United Kingdom) the establishment was ranged against the latter and it was a lonely battle for our hero. "Boltzmann is the last great representative of the atomic theory", declared one critic, probably Wilhelm Ostwald, ironi-

IMAGE
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REASONS

Blue Boltzmann — philosophy, he wrote, "gets on my nerves".

cally considered to be the founder of physical chemistry. Was any prediction ever so ludicrously wrong?

What emerges strongly and perhaps surprisingly from the documents is that the often heated philosophical disputation was not accompanied by any personal ill-will. The protagonists remained friends and Ostwald's advocacy was a major factor in Boltzmann's move from Vienna to the Leipzig faculty in 1900. Boltzmann himself relished the debates and his personal good nature shows forth repeatedly in his correspondence. In a letter to Franz Bretano, for example, he wrote "... nor do I wish or even consider it politeness if someone agrees with me in everything; that is quite clear. I only profit from well-grounded contradiction." His generosity of spirit is particularly apparent in his letters to Svante Arrhenius, a former student who won a Nobel prize in 1905, an honour

that Boltzmann never received despite several nominations. Boltzmann congratulated him on this occasion and, as he had done many times before, wished him success with his future work. No hint of jealousy here, no sign anywhere that lack of recognition contributed to the anguish that led to suicide. In the same vein, Boltzmann's sense of humour comes through in his own writings and in comments by those who knew him. His light-heartedness persisted to the very end, as evidenced for example by his entertaining travelogue *On the Trip of a German Professor into El Dorado*, which describes his journey as visiting professor to the University of California at Berkeley in 1904.

The sadder part of Boltzmann's life emerges in frequent references to his illnesses. He suffered from respiratory ailments, dyspepsia, laryngitis, boils and (according to his wife) urinary tract infections. He himself most often complains of 'neurasthenia', which today might be diagnosed as 'mood disorder', but even the most ardent of Freudians could hardly blame blood in the urine on manic-depressive illness. Nevertheless, he confesses in his letters to periods of depression, confusion and inability to finish work in progress. By the spring of 1906 he was so ill (mentally?) that his lectures were cancelled and colleagues were convinced that he would never return to work. His suicide in September of that year was tragic, but not wholly unexpected by those who knew him. (He had attempted suicide once before when he was unhappy, in Leipzig in 1901.)

Blackmore defines the purpose of his book as being "not to persuade, but to inform, to add new material...". He invites the reader to reach an independent conclusion about Boltzmann's true philosophical persuasion (if, indeed, he had one) and offers none himself. He hints that preoccupation with philosophy may have contributed to Boltzmann's final depression, but rejects the possibility that direct attacks on his own atomic views had anything to do with it.

Did Boltzmann himself leave any clues? Here is a sample to ponder, based on shorthand notes for a philosophy lecture in 1904: "Philosophy gets on my nerves. If we analyze the ultimate ground of everything, then everything finally falls into nothingness. But I have decided to resume my lectures again and look the Hydra of doubt straight in the eye, and it can be quite ominous if one values one's life." □

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Figures of speech

Chris Philippidis

More Than One Mystery: Explorations in Quantum Interference. By Mark P. Silverman. Springer: 1995. Pp. 212. DM48, £19.95 (pbk).

THIS is a beautiful and clear exposition of how quantum interference, non-locality and long-range correlations interweave to produce characteristically quantum effects that have no classical counterparts. From the foundational point of view, however, one has to raise an eyebrow at Silverman's declared intention to proliferate the mysteries of the quantum world. This is an unnecessary blemish.

The term 'quantum wholeness', although ubiquitous in the literature on the foundations of quantum mechanics, is never used in the text. Nevertheless, Silverman develops this theme in a symphonic fashion. Interference, coherence, non-locality and correlations are leitmotifs that get worked and re-worked in a variety of situations and imaginative experiments (some real and some *gedanken*). The unflinching outcome is that the classical intuitions always fail spectacularly.

Through concise but succinct calculations, the author treats several cases involving two-slit interference in conjunction with Aharonov-Bohm solenoids and Einstein-Podolsky-Rosen set-ups. Magnetic fluxes arranged in different topological configurations in various interferometers form the basis of many of his case studies in the earlier part of the book. He then goes on to deal with quantum interference in contexts such as beats in radiative decay of excited atoms, oscillating field spectroscopy and the parallel between optical rotation and a two-level system.

Silverman creatively unmask the features of the quantum domain to reveal the subtle workings of entangled states, second-order correlations, photon bunching and interference in time and so on. He often alludes to foundational issues. During his treatment of interactions of ions with vector potential fields, for example, he raises the question of the inconsistency between the Schrodinger and the Pauli and Dirac formulations.

Yet the book poses an unintended paradox. On the one hand, it successfully develops quantum intuitions and ways of seeing and contrasts them well with their naive or classical counterparts. On the other hand, its language resides within the discourse of the 'mysterious', which undermines the very project of establishing quantum wholeness as a natural mode of expression and communication.

This might have been understandable if the author's communication skills were found wanting. But throughout the book,

Silverman shows considerable awareness of and sensitivity to discursive techniques. The text is well-structured and punctuated by effective rhetorical questions. He also places issues in context by briefly reporting the debates that surround them.

Silverman's mysteries are sustained by a classical and observer-centred narrative, as in:

Can it truly be the case that the AB [Aharonov-Bohm] effect occurs if one looks for two electrons at *one* location, and does not occur if one looks for electrons at *two* locations? If so, the present example leads to an extraordinarily puzzling consequence which I illustrate concretely by resorting to a monochromatic plane-wave description of the electrons.

Having consistently shown the irrelevance of classical imagery, why resort to it when formulating such questions? Why talk of 'looking' at one place or another when the effect discussed result from concrete experimental arrangements? The wiring of detectors to register independently or in coincidence is an integral part of a consistent quantum language.

It would be interesting to know Silverman's views on the de Broglie-Bohm approach. This approach would not only bring out even more forcibly the particular features of quantum mechanics he has so ably demonstrated but would also impose a mystery-free way of expressing them. Perhaps not so good for sales but it would work wonders for quantum physics. □

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Paths of discovery

Douglas Palmer

Dinosaur Tracks and Other Fossil Footprints of the Western United States. By Martin Lockley and Adrian P. Hunt. Columbia University Press: 1995. Pp. 338. \$29.50.

FROM their publication record, Martin Lockley and Adrian Hunt would seem to have invented a subsistence of 'dino-trekking'. They have certainly done a very good job in promoting the scientific investigation of fossil dinosaur tracks. They build though on a respectable tradition that, in the United States, goes back to Edward Hitchcock's mid-nineteenth-century pioneering studies of the Jurassic period of the Connecticut Valley. Hitchcock regarded the three-toed imprints he found there as the footprints of giant birds. He was spectacularly wrong of course, but the ornithischian characters of some dinosaurs had not been recognized

at that time. This historical digression does, however, illustrate the sort of pitfalls lying in the stratigraphic record awaiting the unwary ichnologist.

Lockley and Hunt do not balk from these and the many other problems that beset their area of study, such as the taxonomy of trace fossils. Rarely can tracks be linked to body fossils, so there has to be an independent nomenclature. The difficulties of matching prints with their biological maker are not confined to species or genera but can still cause problems for the experts even at the class level. For instance, the authors' discussions of Upper Palaeozoic tracks highlight the difficulty of distinguishing between amphibian and reptile prints.

This non-dinosaur aspects of the book are particularly interesting, partly because the dinosaurs are so well served in other books (such as D. D. Gillette and M. G. Lockley's *Dinosaur Tracks and Traces*, Cambridge University Press, 1990) and the non-dinosaur literature is so scattered. But these trace fossils also promise significant progress in our understanding of early tetrapods and the environments in which they lived. If it is necessary for this area of study to 'piggyback' on the more popular appeal of dinosaurs, so be it.

The Cenozoic tracks of birds and mammals also get a look in but have far fewer recorded sites in the western states than the reptiles do in the older rocks. Clearly this is not a lack of suitable terrestrial sedimentary rocks, which are abundant on the wide flanks of the Rockies. They just haven't been found yet. Every other page of the book refers to old collections that need reworking or new collections that are awaiting study.

From the subtitle, the book might seem a bit 'parochial'. But the region covered is a large parish and probably contains as many sequences of terrestrial sediments, through as much of the Phanerozoic, as anywhere on Earth. There are enough tracks and trails here to fill many a PhD thesis into the next millennium. □

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New in paperback

The World of Physical Chemistry by Keith J. Laidler. Oxford University Press, £25. "Laidler has provided a masterly survey of the field, which will help to put the record straight". Peter Atkins, *Nature* **365**, 615 (1995).

The Elm and the Expert: Mentalese and its Semantics by Jerry A. Fodor. MIT Press, £8.95, \$10. Reviewed by Stephen Pinker in *Nature* **368**, 360 (1994).

Making Science: Between Nature and Society by Stephen Cole. Harvard University Press, £10.50. Reviewed by S. Blume in *Nature* **364**, 725 (1993).

Increased biological productivity and export production in the glacial Southern Ocean

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A range of complementary radionuclide proxies in sediments of the southernmost Atlantic Ocean over the past 140,000 years indicate that glacial periods were characterized by greatly increased fluxes of biogenic detritus out of surface waters. This increase in export production, which may have contributed to lower concentrations of carbon dioxide in the glacial atmosphere, was accompanied by more than a fivefold increase in accumulation of lithogenic iron transported by winds from Patagonian deserts. These observations support the hypothesis that the iron limitation of today's Southern Ocean productivity was relieved in glacial periods by a greater supply of iron from wind-blown dust.

MARTIN and co-workers¹⁻³ noted that high-nutrient low-chlorophyll (HNLC) waters tend to coincide with regions of particularly low aeolian supply of lithogenic dust, the principal source of biologically available iron in regions far from continental or shallow-water sedimentary sources⁴. The Southern Ocean, a present-day HNLC region, fits this pattern, with typical aeolian iron fluxes ($\sim 1 \text{ mg m}^{-2} \text{ yr}^{-1}$) among the lowest in the world oceans⁵. Martin¹ postulated that the biologically available iron associated with the 15- to 50-fold increase in deposition of dust, as recorded in glacial-age sections of Antarctic ice cores^{6,7}, could have relieved the iron deficiency that at present limits biological productivity and biomass. Martin further postulated that this relief from iron limitation enabled phytoplankton to consume nearly all of the nutrients that upwelled into Southern Ocean surface waters. In the present-day ocean, these nutrients are advected unused back into the deep sea pre-formed nutrients. Complete consumption of nutrients in Antarctic surface waters, coupled with the export of biogenic detritus to the deep sea, would have effected the partitioning from the atmosphere to the deep ocean (as dissolved inorganic carbon) an amount of carbon more than sufficient⁸⁻¹⁰ to have caused the lower (by $\sim 80 \text{ p.p.m.}$) atmospheric CO_2 concentrations that existed during glacial periods^{11,12}.

Direct evidence for iron limitation of modern ocean productivity was provided by an *in situ* iron enrichment study¹³ in the eastern Equatorial Pacific Ocean (a HNLC region) where investigators observed post-enrichment increases in primary production rates, in biomass, and in the efficiency of photochemical energy conversion^{13,14}. Indirect evidence for iron fertilization of HNLC waters can be inferred from observations of persistent patches of elevated chlorophyll concentrations in areas downstream of iron sources, both in the Equatorial Pacific¹³ and in the Southern Ocean¹⁵. On a smaller scale, primary production rates and phytoplankton biomass were found to correlate positively with iron concentration within the Antarctic polar front zone¹⁶.

Despite accumulating evidence for iron limitation in the modern ocean, palaeoceanographic studies of the Southern Ocean have not revealed evidence for greatly increased rates of primary

production during glacial times. Proxies of surface-water nutrient concentrations have failed to provide unambiguous evidence for the reduced nutrient levels in the glacial Southern Ocean¹⁷⁻²⁰ that would have accompanied iron fertilization if all other conditions had remained unchanged. Burial of biogenic carbonate in Southern Ocean sediments declined substantially during glacial times²¹, while the regional-average burial flux of opal, the principal biogenic phase in Southern Ocean sediments, appears to have remained relatively constant^{22,23}.

Burial rates of biogenic detritus may not provide reliable monitors of past ocean productivity²⁴, however, because most of the opal, carbonate and organic carbon settling onto the sea bed are remineralized rather than buried. Preservation of biogenic phases is highly variable in space and time, clearly evident for opal preservation in modern Southern Ocean sediments²⁵. Furthermore, preservation efficiency cannot be reconstructed from sedimentary records. Consequently, we have turned to proxy tracers which are independent of burial/remineralization ratios of biogenic material, thereby providing a more robust test for past changes in the primary productivity of the Southern Ocean. New proxy records, presented here, indicate a dramatic increase in export production of subantarctic waters during glacial periods. Increased fluxes of iron coincided with periods of increased export production, implicating iron fertilization as a probable factor responsible for increased export production.

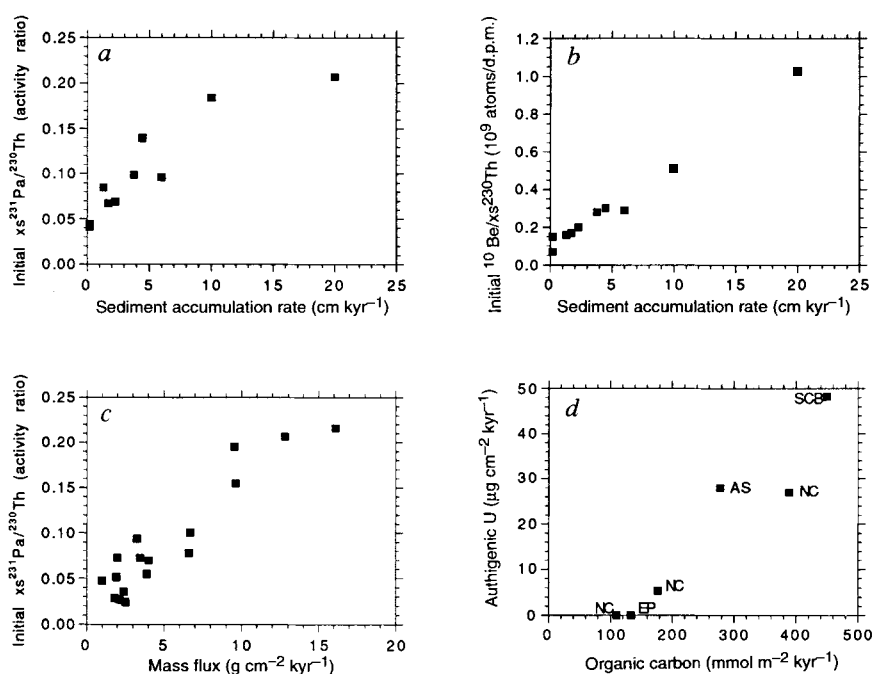
New proxies for export production

Export production is defined as the flux of biogenic detritus that escapes remineralization in the euphotic zone and sinks into the deep sea. It is the balance between the upwelling of inorganic nutrients and CO_2 into surface waters and the sinking of particulate biogenic material into the deep sea that determines the efficiency of the biological pump in partitioning CO_2 between the ocean and the atmosphere. Consequently, we look to proxies that track features of export production, rather than gross (or total) primary production, to assess past responses of the Southern Ocean to climate change.

In open-ocean regions far from continental sources of lithogenic material, particulate matter sinking through the water column is predominantly of biogenic origin²⁶. Because the deep-water flux of biogenic particles scales directly with export production²⁷, a proxy that monitors particle flux will provide a record of past changes in export production, provided that the proxy is well preserved in the sediments.

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FIG. 1 Calibration of new proxies used to evaluate past changes in Southern Ocean productivity as expressed through positive relationships with particle flux (or equivalent sediment accumulation rate) or with the flux of particulate organic matter. **a**, Initial unsupported (designated as "XS" to indicate the amount of each isotope present in excess of that which is supported by its uranium progenitor) $^{231}\text{Pa}/^{230}\text{Th}$ ratios plotted against sediment accumulation rate for sediments representing a transect across the North Pacific Ocean at $\sim 40^\circ\text{N}$ (replotted from ref. 45). These results represent a wide range of conditions, from pelagic red clays of the central north Pacific gyre (minimum accumulation rates) to sites in the northwestern Pacific located downwind from the principal sources of Asian dust (intermediate accumulation rates) to productive waters of the California Current (maximum accumulation rates). **b**, Initial $^{10}\text{Be}/^{230}\text{Th}$ ratios plotted against sediment accumulation rate for the same set of cores shown in **a**; data replotted from ref. 45. **c**, Initial unsupported $^{231}\text{Pa}/^{230}\text{Th}$ ratios plotted against mean annual mass flux of particles collected by sediment traps (replotted from ref. 31). Results from sediment traps at one site identified³¹ as suffering from an undertrapping bias have been excluded from this figure. **d**, Accumulation rate of authigenic uranium in Holocene sediments plotted against mid-water-column flux of particulate organic carbon. Sources of data for **d**: NC, transect of three stations normal to the coast of northern California from productive waters of the California Current seaward to the oligotrophic gyre (U data replotted from ref. 45; original carbon flux data presented in ref. 53); EP, US-JGOFS equatorial Pacific site (0° , 140°W) (carbon flux data from ref. 54; U accumulation (R.F.A., unpublished data)); AS, western Arabian Sea (carbon flux data from ref. 55; U accumulation (R.F.A., and F. Sirocko, unpublished data)); SCB, San Clemente basin, off southern California (carbon flux is the flux of labile organic carbon to the sea bed at $\sim 1,900\text{ m}$ (ref. 56); U accumulation (R.F.A., unpublished data)).



Scavenging of particle-reactive elements from the ocean responds systematically to changing particle flux. Protactinium-231 and ^{230}Th are produced uniformly throughout the ocean by radioactive decay of dissolved uranium isotopes. Both ^{231}Pa and ^{230}Th are removed quantitatively from sea water by scavenging to particulate matter but, because of the longer residence time of ^{231}Pa , one finds in the modern ocean that particulate $^{231}\text{Pa}/^{230}\text{Th}$ ratios rise with increasing particle flux^{28, 31} (Fig. 1a, c). An analogous radionuclide-ratio proxy can be constructed from the ^{10}Be – ^{230}Th pair. Although ^{10}Be is a cosmogenic nuclide produced in the atmosphere³², its distribution within an ocean basin is relatively uniform, reflecting its relatively long mean oceanic residence time with respect to scavenging (500–1,000 yr; refs 33, 34). Because the residence time of ^{10}Be is longer than that of ^{230}Th , $^{10}\text{Be}/^{230}\text{Th}$ ratios, like $^{231}\text{Pa}/^{230}\text{Th}$ ratios, rise with increasing particle flux (Fig. 1b), from minimum values in oligotrophic central ocean gyres to maximum values at ocean margins, where upwelling-induced productivity and continental erosion together create a high particle-flux regime of intense scavenging. Even the most soluble of these nuclides (^{10}Be) is not affected significantly by remineralization during early diagenesis at the sea bed³⁵, thereby eliminating variable preservation as a factor influencing the sedimentary record. Assuming, then, that the relationships shown in Fig. 1a–c have remained constant through time, down-core profiles of radionuclide ratios can be used to monitor past variability of export production^{36, 38}, at least in a qualitative manner.

Sedimentary uranium concentrations provide a proxy that responds to the flux of organic carbon delivered to the sea bed. Uranium, which exists as a relatively non-reactive dissolved hexavalent species in the presence of oxygen, is precipitated as an amorphous tetravalent phase under anoxic chemically reducing conditions³⁹ that occur in sediments when the flux of organic carbon to the sea bed exceeds the supply of molecular oxygen by diffusion from bottom waters. Below the threshold flux of organic carbon required to create anoxic conditions in the sediments, there is no significant deposition of authigenic uranium.

unpublished data); AS, western Arabian Sea (carbon flux data from ref. 55; U accumulation (R.F.A., and F. Sirocko, unpublished data)); SCB, San Clemente basin, off southern California (carbon flux is the flux of labile organic carbon to the sea bed at $\sim 1,900\text{ m}$ (ref. 56); U accumulation (R.F.A., unpublished data)).

Above this threshold, there a positive, though nonlinear, relationship between organic carbon flux and the accumulation rate of authigenic uranium^{40, 41}. These principles are illustrated in Fig. 1d, which shows that even in pelagic regions of moderately high export production, such as the Equatorial Pacific Ocean, the present flux of organic carbon to the sea bed lies below the threshold to induce authigenic uranium precipitation. In the modern ocean, significant burial of authigenic U is found in areas of high export production, such as occur in the ocean-margin upwelling regions off California and in the western Arabian Sea. Assuming that a similar relationship existed in the past, down-core records of authigenic U can be used to constrain past changes in the flux of carbon to the deep sea.

Southern Ocean setting

The Southern Ocean, defined here as the region to the south of the Subtropical Convergence (STC), can be divided into two zones: the subantarctic zone, lying between the STC and the Antarctic polar front (APF), and the Antarctic zone to the south of the APF (Fig. 2). Surface waters of both zones are rich in major inorganic nutrients (Fig. 2). A meridional segregation of principal planktonic species assemblages is reflected in the composition of the underlying sediments. Modern sediments of the subantarctic zone are characteristic of oligotrophic subtropical gyres of the Atlantic Ocean, and reflect the predominance of carbonate-secreting plankton ($\sim 80\%$ carbonate, $\sim 15\%$ terrigenous and $\sim 5\%$ opal at the location of V22-108; Fig. 2²³). Modern sediments within, and south of, the APF zone are siliceous oozes ($\sim 75\%$ opal, $\sim 20\%$ terrigenous and $\sim 5\%$ carbonate at the location of RC13-259; Fig. 2²³), reflecting the prominence of diatoms in Antarctic waters.

Systematic changes in sediment composition occurred throughout the Southern Ocean in response to changing climate conditions. In the subantarctic zone, opal accumulation rates increased several-fold during glacial periods^{22, 23}, whereas carbonate accumulation rates declined sharply^{21, 42}. Glacial Antarctic sediments are characterized by the virtual elimination of

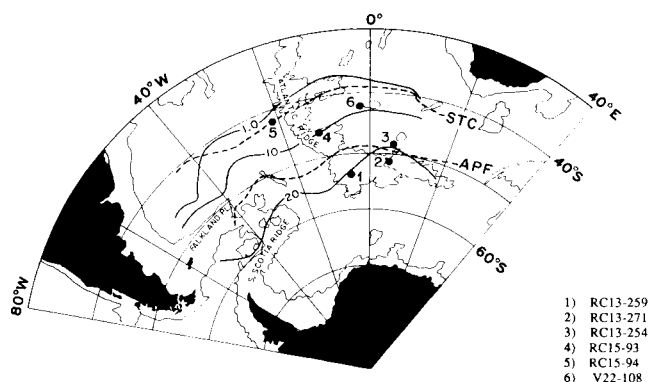


FIG. 2 Map of the Atlantic sector of the Southern Ocean showing locations of cores examined in this study. Solid contours are isolines of surface water nitrate concentration (μM) during austral summer (from data base of T. Takahashi, containing observations from 1932 to the present), and illustrate the abundance of nutrients in surface waters of the modern subantarctic zone. Within the region from which the cores were collected ($\sim 25^\circ\text{W}$ to $\sim 10^\circ\text{E}$) the mean position of the Subtropical Convergence (dashed contour) is at $\sim 40^\circ\text{S}$, while the mean position of the Antarctic polar front (dashed contour) is at $\sim 50^\circ\text{S}$.

carbonate²¹, accompanied by a decline in opal accumulation rates, suggesting lower productivity of glacial Antarctic waters^{22,23}. In this study, we have examined records from six cores (Fig. 2): two in the northern subantarctic zone (V22-108, RC15-94), two in the southern subantarctic zone (RC15-93, RC13-254), and two in the Antarctic zone (RC13-271, RC13-259).

Glacial-interglacial changes in export production

In a preliminary report on the results of this study³⁶, we showed that the $^{231}\text{Pa}/^{230}\text{Th}$ particle-flux proxy record in subantarctic core RC13-254 is a 'mirror image' of that in Antarctic core RC13-259 (core locations shown in Fig. 2). Compared to modern conditions, these records show that export production of Antarctic waters was reduced during glacial periods, whereas export production increased in the subantarctic zone. Here we show additional proxy records from RC13-254 to better characterize changes in export production, and to demonstrate the relationship between these changes and global climate variability. Following that, we will compare Holocene (past 10 kyr) conditions recorded in six cores with average conditions during the Last Glacial Maximum (LGM; roughly 16–26 kyr before present, BP) to illustrate regional patterns of change.

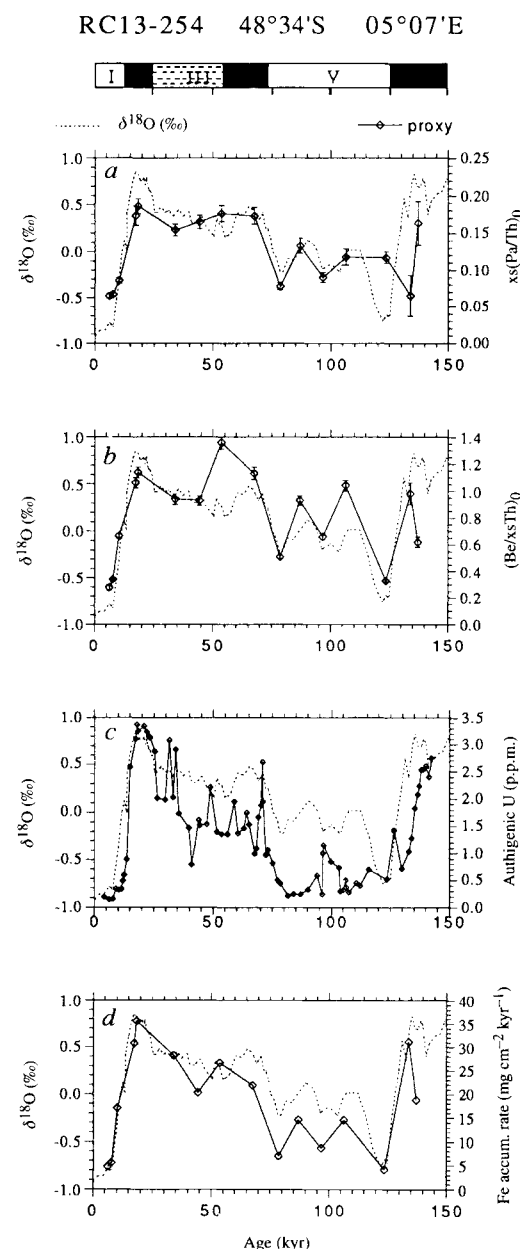


FIG. 3 Down-core profiles from core RC13-254 plotted against age. The age model for this core is from ref. 23, except that the model for the period from 20 kyr BP to the present has been modified on the basis of recent ^{14}C results (R.F.A. and N.K., unpublished data). The scale bar distinguishes interglacial (white) and glacial (black) climate stages (stage boundaries are defined by the maximum rate of change in the ^{18}O record), as well as an intermediate stage (dashed-line 'shading'). A normalized (relative to an average $\delta^{18}\text{O}$ set to 0‰) SPECMAP $\delta^{18}\text{O}$ record⁴³ is also drawn in each panel to illustrate the coherence between proxy records and climate variability ($\delta^{18}\text{O}$ increases with increasing volume of continental ice sheets). a, Initial (decay-corrected) unsupported (xs) $^{231}\text{Pa}/^{230}\text{Th}$ activity ratio (measured by isotope dilution alpha spectrometry³⁰). b, Ratios of initial (decay corrected) $^{10}\text{Be}/\text{xs}^{230}\text{Th}$ (^{10}Be measured by accelerator mass spectrometry³⁰). c, Concentration of authigenic uranium (U_A), which is computed from measured total concentrations of U and Th as (U_A (p.p.m.) = U_T (p.p.m.) - $0.23 \times \text{Th}_T$ (p.p.m.)); subscripts "A" and "T" represent "authigenic" and "total", respectively. d, ^{230}Th -normalized accumulation rate⁴⁷ of total iron (measured by direct current plasma emission spectrometry⁴²). The ^{230}Th -normalization method is used to correct for sediment focusing by deep ocean currents. Tick marks on the scale bar correspond to intervals of 25,000 years.

Both particle-flux proxies ($^{231}\text{Pa}/^{230}\text{Th}$ and $^{10}\text{Be}/^{230}\text{Th}$ ratios) record the increased export production of the subantarctic zone that existed during glacial periods (Fig. 3a, b). A normalized SPECMAP $\delta^{18}\text{O}$ curve⁴³ is plotted with each proxy record to show that changes in export production were coherent with global climate change. In particular, a pronounced decrease in export production coincided with the transition from the LGM to the Holocene. Comparison of the absolute values of the particle-flux proxies during the LGM (Fig. 3a, b; $^{231}\text{Pa}/^{230}\text{Th} \approx 0.2$, $^{10}\text{Be}/^{230}\text{Th} \approx 1.0$) with modern analogues (Fig. 1) suggests that export production during the LGM may have been as great as that occurring today in productive ocean-margin regions. During the transition from the LGM to the Holocene, the radionuclide ratios decrease to values (Fig. 3a, b; $^{231}\text{Pa}/^{230}\text{Th} \approx 0.06$, $^{10}\text{Be}/^{230}\text{Th} \approx 0.25$) nearly as low as occur today in central ocean gyres (Fig. 1), indicating a substantial reduction in export production.

Burial of authigenic U (U_A) in glacial-age sediments of RC13-254 (Fig. 3c) is consistent with the increased export production inferred from the particle-flux proxies. Whereas the concentration of U_A in Holocene sediments is close to our detection limit,

there is an abrupt increase in U_A concentration going back to the LGM. Although maximum concentrations (and accumulation rates⁴²) of U_A are found during the LGM, suggesting that export production was greatest during periods of maximum ice volume, U_A is abundant in all glacial-age sediments (~15–75 kyr BP), indicating that the flux of organic carbon to the sea bed exceeded the threshold to induce uranium precipitation throughout this interval. This pattern is repeated going back in time, where sediments deposited during the last interglacial (~80–130 kyr) have much lower concentrations of U_A than occur in sediments from the penultimate glacial maximum (~140 kyr BP), indicating a regular pattern of increased export production during glacial periods.

Particle-flux proxies indicate that export production was greater during the LGM than under modern conditions across the subantarctic zone (Fig. 4a, b). Maximum values for both proxies occur in the southern subantarctic zone (45°–50° S), where values ($^{231}\text{Pa}/^{230}\text{Th} \approx 0.2$, $^{10}\text{Be}/^{230}\text{Th} \approx 1.1$) are comparable to those occurring today in productive ocean-margin regions (Fig. 1). In the northern subantarctic zone (~43° S), $^{10}\text{Be}/^{230}\text{Th}$ ratios (Fig. 4b) were substantially greater during the LGM than during the Holocene, but not as large as in the southern subantarctic zone, suggesting a northward gradient of decreasing export production within the subantarctic zone. The $^{231}\text{Pa}/^{230}\text{Th}$ ratios are consistent with this northward decrease in export production during the LGM, showing relatively little change between LGM and Holocene values in the northern subantarctic zone, in contrast to the difference of more than a factor of two in the southern subantarctic cores (Fig. 4a).

Records provided by the two radionuclide-ratio proxies of particle flux (Fig. 4a, b) are not entirely redundant because these ratios are influenced by secondary factors in addition to particle flux. For example, it has been suggested that ^{231}Pa exhibits a stronger affinity for scavenging by biogenic opal than by other particulate phases^{31,44}. If true, then this preference may contribute to high $^{231}\text{Pa}/^{230}\text{Th}$ ratios in Holocene sediments in our southernmost core (RC13-259; up to 80% opal in Holocene sediments) and to the low $^{231}\text{Pa}/^{230}\text{Th}$ ratios in LGM sediments in the northernmost cores (Fig. 4a), where opal accumulation rates (Fig. 4d) and concentrations⁴² remain low throughout the record. Scavenging of ^{10}Be is not affected by the opal content of sinking particles^{30,45}, so the uniform increase of the $^{10}\text{Be}/^{230}\text{Th}$ ratio across the subantarctic zone during the LGM (Fig. 4b) may reflect, more directly, the glacial increase in export production.

Authigenic uranium records (Fig. 4c) are consistent with the particle-flux proxies, indicating a substantial increase in delivery of organic carbon to the subantarctic sea bed during the LGM. Whereas organic carbon fluxes are generally below the threshold to initiate U_A burial in Holocene subantarctic sediments, accumulation rates of U_A in LGM sediments across the subantarctic zone (5–30 $\mu\text{g cm}^{-2} \text{ kyr}^{-1}$; Fig. 4c) are comparable to those found today in productive ocean-margin regions (Fig. 1d). Glacial-age U_A records are also consistent with the particle-flux proxies in indicating a northward decrease in export production within the subantarctic zone, in that we find rates of U_A accumulation two to three times greater in the southern subantarctic zone than in the northern subantarctic cores (Fig. 4c).

Anoxic conditions required for precipitation of U_A in sediments may be created by reduced bottom-water oxygen concentrations as well as by increased fluxes of organic carbon to the sea bed. We rule out low bottom-water oxygen concentrations as the primary factor driving burial of U_A because, at sites well to the south of the APF, sediments deposited during the LGM lack any U_A . This is true not only for RC13-259, included in this study, but also for other cores from the Atlantic⁴⁶, Indian^{31,41} (G. Bareille, personal communication) and Pacific (R.F.A., unpublished data) sectors of the Southern Ocean. If Circumpolar Deep Water had been uniformly depleted in oxygen during the LGM, then one would expect to find U_A in LGM sediments throughout the Southern Ocean. The fact that U_A in LGM sedi-

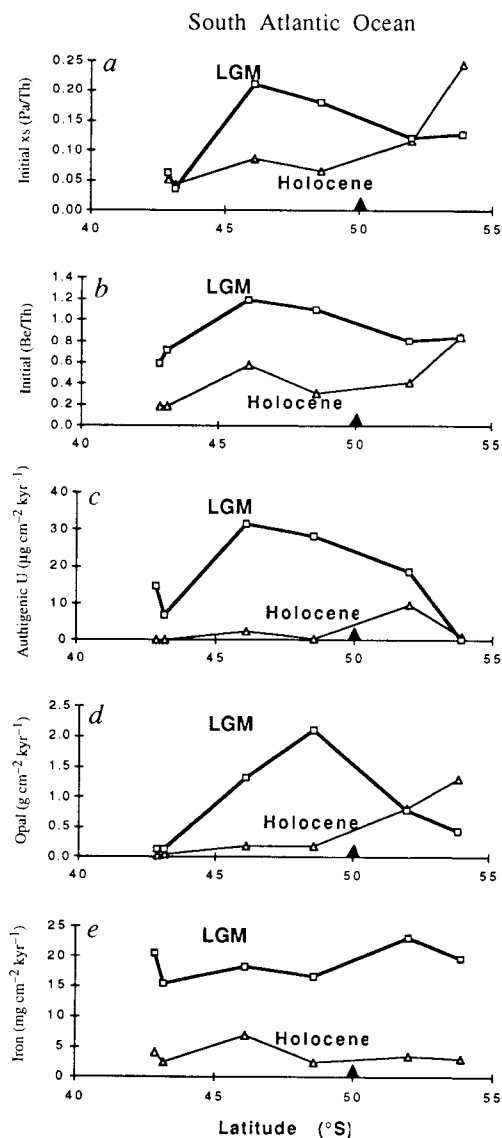


FIG. 4 a, Palaeoceanographic records from six cores (locations shown in Fig. 2) recovered from the Atlantic sector of the Southern Ocean. Average values for Holocene (0–10 kyr ago) and Last Glacial Maximum (16–26 kyr) sections of each core are plotted against latitude. The filled triangle marks the mean latitude of the modern Antarctic polar front, separating Antarctic and subantarctic zones. a, Initial (decay corrected) unsupported (xs) $^{231}\text{Pa}/^{230}\text{Th}$ ratios; b, initial $^{10}\text{Be}/\text{xs } ^{230}\text{Th}$ ratios; c, accumulation rate of authigenic uranium; d, ^{230}Th -normalized (see Fig. 3 legend) flux of opal (opal concentrations are taken from ref. 23); e, ^{230}Th -normalized flux of iron. Authigenic uranium accumulation rates are derived from linear sediment accumulation rates interpolated between age horizons²³, corrected to mass accumulation rate using *in situ* density. Because U is added to sediments in dissolved form, by diffusion, rather than removed from the water column by particles, it is inappropriate to use the ^{230}Th -normalization technique to evaluate authigenic U accumulation rates.

ments is largely restricted to the subantarctic zone (and, possibly, within the region of the APF), where the particle-flux proxies also indicate a greatly increased export production during the LGM, supports the conclusion that the U_A records are influenced primarily by past changes in carbon flux, rather than by bottom-water oxygen concentrations.

Each proxy of export production may respond to secondary factors, thereby permitting multiple interpretations of any individual record. The strength of our study lies in the fact that we have three independent proxies ($^{231}\text{Pa}/^{230}\text{Th}$ ratios, $^{10}\text{Be}/^{230}\text{Th}$ Th

ratios and the burial of authigenic uranium) that all indicate a substantial increase in export production in the subantarctic zone during glacial periods. Furthermore, by comparison with modern analogues, these proxies all suggest that the level of export production in the southern subantarctic zone during the LGM was comparable to conditions that exist today in highly productive ocean-margin regions.

Decoupling of organic carbon and opal records

Previous studies^{22,23} have concluded that the increase in opal accumulation in Antarctic sediments approximately balanced the decline in opal accumulation in subantarctic sediments during transition from glacial to interglacial periods. This was interpreted to imply little net change in export production of the Southern Ocean as a whole. Re-examining a set of cores from the South Atlantic, we find evidence for larger rates of opal burial, overall, during peak glacial conditions (Fig. 4d). Our evaluation differs from those in previous studies in that we use the ²³⁰Th-normalization approach⁴⁷ to correct for focusing of sediments by bottom currents. Recognizing the limitations imposed by the small number of cores in our study, integrating over the zone covered by our samples we find that opal burial was greater during the LGM by roughly a factor of two (Fig. 4d).

Authigenic uranium records (Fig. 4c), supported by the radionuclide-ratio proxies, indicate an enhancement of organic carbon flux in the glacial Southern Ocean substantially larger than the twofold increase in opal burial rate, implying a decoupling, or nonlinearity, of the relationship between opal burial and carbon flux. That such a nonlinearity exists can be demonstrated as follows. During the LGM, uranium records indicate high rates of organic matter delivery to northern subantarctic sediments (Fig. 4c), at a time when the organic carbon flux to Antarctic sediments (RC13-259) was below the threshold to induce U_A burial. Despite having much lower organic carbon fluxes, opal accumulation rates at the Antarctic site of RC13-259 exceeded rates of opal accumulation in the northern subantarctic (RC15-94, V22-108) by more than a factor of three (Fig. 4d).

Comparing sediments deposited at different times provides a more quantitative illustration of the nonlinear relationship between opal accumulation and carbon flux. Whereas the Holocene rate of opal accumulation at the Antarctic site of RC13-259 ($\sim 1.2 \text{ g cm}^{-2} \text{ kyr}^{-1}$) exceeds the rate of opal accumulation at the northern subantarctic sites during the LGM ($0.12\text{--}0.13 \text{ g cm}^{-2} \text{ kyr}^{-1}$) by a factor of ~ 10 (Fig. 4d), U_A accumulation in northern subantarctic sediments during the LGM ($7\text{--}14 \mu\text{g cm}^{-2} \text{ kyr}^{-1}$) exceeded the Holocene rate of U_A accumulation at the site of RC13-259 ($\sim 1.1 \mu\text{g cm}^{-2} \text{ kyr}^{-1}$) by roughly an order of magnitude (Fig. 4c). Combining these two inequalities produces U_A /opal ratios that decrease from glacial northern subantarctic sediments to Holocene Antarctic sediments by two orders of magnitude. Although the accumulation rate of U_A is not a linear function of carbon flux, there is, nevertheless, a positive relationship between the two parameters⁴¹. Consequently, these inequalities indicate that (carbon flux), (opal accumulation) ratios vary in space and time, certainly by more than an order of magnitude, and possibly by as much as two orders of magnitude.

Factors creating this nonlinearity remain unknown. Either greatly reduced opal preservation in the subantarctic zone during the LGM, or an ecological shift of dominant species assemblages away from diatoms to phytoplankton without shells, could contribute to the observed decoupling between carbon flux and opal accumulation in the Southern Ocean. Large spatial variability of opal preservation in the modern Southern Ocean²⁵ suggests that the first possibility could be an important factor. The latter possibility can be tested by analysing sedimentary organic matter for molecular biomarkers (for example, pigment composition). Whatever the cause, this decoupling of carbon flux from opal

burial explains the failure of previous studies^{22,23} using opal accumulation to find evidence for greatly enhanced export production in the glacial Subantarctic Ocean.

Iron fertilization by aeolian dust

Fluxes of iron to glacial sediments in the Atlantic sector of the Southern Ocean were greater than today (Holocene) by more than a factor of five (Figs 3d and 4e). The pattern of iron accumulation in RC13-254 is remarkably similar to the global ice volume record (Fig. 3d), as well as to the down-core records of the proxies of export production (Fig. 3a–c), suggesting that supply of iron, like export production, varied in response to past changes in Earth's climate. The potential for fertilization of iron-limited waters depends on the source of this iron, with iron supplied in the form of atmospheric dust more likely to be biologically available than iron from other sources (lithogenic material transported by deep-ocean currents, for example). Location of these sites far from the continents (Fig. 2) eliminates fluvial supply as a significant source. The glacial flux of iron to a site perched high on the Mid-Atlantic Ridge (RC15-93, $\sim 46^\circ \text{S}$, 2,714 m) is similar to fluxes recorded by deeper cores, both to

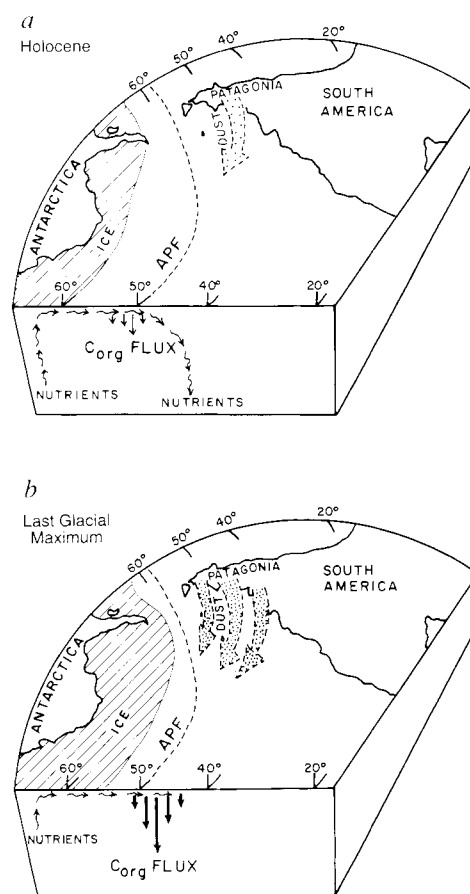


FIG. 5 Schematic representation of the model proposed to explain patterns of export production in South Atlantic Ocean over glacial-interglacial timescales. *a*, During the Holocene, the zone of maximum export production is south of the Antarctic polar front (APF). Lower supply of aeolian dust, the primary source of dissolved Fe in the surface waters of the region, causes phytoplankton to be Fe-limited. Much of the upwelled nutrients are returned to depth as pre-formed nutrients. *b*, At Last Glacial Maximum, the zone of maximum export production was shifted northwards, to the north of present-day location of the APF (the dashed line in the figure marks the location of present-day APF; the location of the APF during glacials is not known). Increased supply of aeolian dust relieved iron limitation, enabling phytoplankton to consume nutrients more completely, and caused the export production of particulate organic carbon (C_{org}) and related biogenic phases to be significantly greater than during the Holocene.

the north (for example, V22-108, $\sim 43^\circ$ S, 4,171 m) and to the south (RC13-254, $\sim 48^\circ$ S, 3,636 m), ruling out a change in supply of lithogenic material either by bottom currents or by distal turbidites. If ice rafting supplied the additional lithogenic material deposited during glacial times, then we would expect deposition of large ice-rafted debris (IRD) fragments to parallel the relatively uniform glacial flux of iron (Fig. 4e). Contrary to this expectation, glacial fluxes of large IRD fragments decrease from south to north by more than a factor of 200 among the cores studied⁴⁸, indicating that the principal source of the additional lithogenic material supplied to the subantarctic region during glacial times was not ice rafting. Ruling out other sources, we thus tentatively conclude that the enhanced glacial iron flux is dominantly aeolian and therefore passed through the surface ocean *en route* to the sea bed.

The composition of glacial dust in Antarctic ice recovered from Dome C points to a unique provenance from the glacial deserts of Patagonia⁴⁹ immediately upwind from our study region in the core of the Westerlies (Fig. 5). Based on the proximity of this source, we suggest that the increased flux of lithogenic material in glacial South Atlantic sediments was largely due to an increased flux of dust ablated from Patagonia. Biologically available iron associated with this glacial-age dust would have relieved iron limitation in this present-day HNLC region, as postulated by Martin and co-workers^{1,2}.

If iron fluxes increased throughout our study region during glacial times (Fig. 4e), then why did export production decline during the LGM at the site of our southernmost core (RC13-259)? Two reasonable explanations can be offered. One is that sea ice cover south of the APF was so extensive during glacial times that primary production was inhibited, even during summer^{23,48,50}. The other explanation is that little Patagonian dust was transported this far south and that the lithogenic material south of the APF is primarily IRD, which may have released little biologically available iron into surface waters. Although neither explanation can be fully tested with existing data, lithogenic phases in RC13-259 do have a composition that is distinctly different from those at sites further to the north⁴², indicating a different source for lithogenic material at the southern limit of our study region, and thereby favouring the second explanation or, at least, some combination of the two explanations.

Implications for atmospheric CO₂ regulation

Patterns of export production similar to those shown here have been documented recently for the Indian sector of the Southern Ocean^{41,51}. Although these studies have relied primarily on the sedimentary record of authigenic uranium, both the magnitude and phase relationship of uranium burial are comparable to those of the South Atlantic (U_A accumulation rates $>20 \mu\text{g cm}^{-2} \text{ kyr}^{-1}$ in subantarctic sediments, peaking during glacial periods; lower rates in Antarctic sediments with maximum accumulation during interglacials). Even without supporting evidence from the radionuclide-ratio proxies of particle flux, one can infer by analogy with the South Atlantic that export production in the subantarctic zone of the Indian sector of the Southern Ocean rose during glacial periods to levels found today in productive ocean-margin regions. Future studies must examine records from the South Pacific to determine if enhanced export production was a circumpolar feature of the glacial Southern Ocean.

Martin postulated¹ that iron fertilization stimulated the efficiency of the biological pump throughout the glacial Southern Ocean. Martin's original hypothesis finds substantiation, however, only in the subantarctic zone. For one reason or another, glacial productivity was lower in Antarctic waters well to the south of the Antarctic polar front. Consequently, whereas iron fertilization of the Southern Ocean may have contributed significantly to the reduced concentrations of CO₂ in the glacial atmosphere, the effect would have been less than originally postulated. For example, a box-model simulation⁵² suggests that the complete stripping of nutrients from subantarctic surface waters would reduce atmospheric CO₂ by 15–30 p.p.m. Although the actual contribution to the glacial reduction of atmospheric CO₂ would depend on unquantifiable variables, such as the extent of ice cover, changes in circulation patterns, and upwelling rates, the range of CO₂ reduction produced by the model is well below the observed reduction of ~ 80 p.p.m. (refs 11, 12). Nevertheless, our results demonstrate that the biogeochemical principles embodied within Martin's¹ 'iron hypothesis' find validation in nature. When the glacial environment provided a sustained supply of iron, in the form of dust, to a broad expanse of present-day HNLC waters, the result was an explosion of biological productivity, accompanied by fluxes of particulate biogenic debris, including carbon, to the deep sea of a magnitude that finds its closest modern analogue in highly productive ocean-margin regimes. □

Received 7 April; accepted 17 November 1995.

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ACKNOWLEDGEMENTS. We thank R. Barber and R. Francois for constructive comments as well as T. Takahashi and S. Sutherland for access to, and assistance with, their Southern Ocean data base. This work was supported by the US National Science Foundation.

Crystal structure of the RAR- γ ligand-binding domain bound to all-*trans* retinoic acid

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The 2.0-Å crystal structure of the ligand-binding domain (LBD) of the human retinoic acid receptor (RAR)- γ bound to all-*trans* retinoic acid reveals the ligand-binding interactions and suggests an electrostatic guidance mechanism. The overall fold is similar to that of the human RXR- α apo-LBD, except for the carboxy-terminal part which folds back towards the LBD core, contributing to the hydrophobic ligand pocket and 'sealing' its entry site. We propose a 'mouse trap' mechanism whereby a ligand-induced conformational transition repositions the amphipathic α -helix of the AF-2 activating domain and forms a transcriptionally active receptor.

THE three retinoic acid (RAR) and three retinoid X receptors (RXR) (α , β and γ , and their isoforms) belong to the nuclear receptor superfamily and act as ligand-inducible transcriptional regulators transducing the pleiotropic effects of retinoic acids on morphogenesis, differentiation and homeostasis during development of the embryo and postnatal life. RARs are activated by both isomers of RA, all-*trans* (T-RA) and 9-*cis* (9C-RA), whereas RXRs bind to and are activated only by 9C-RA. Although RARs and RXRs can form homodimers, heterodimerization increases the efficiency of binding to the cognate response elements (refs 1–5 and references therein).

Nuclear receptors display a modular structure with six distinct regions (denoted A–F) with evolutionarily conserved DNA (DBD; region C) and ligand-binding (LBD; region E) domains⁶. The structural basis of DNA recognition is well established from biochemical, nuclear magnetic resonance (NMR), and X-ray crystallography analyses (refs 7–10 and therein), but much less is known about the LBD which upon ligand binding acts as a molecular 'switch' controlling multiple receptor functions, including transactivation and AP1 transrepression (refs 1–5, 11, 12 and therein). Region E has two additional functional domains which overlap the LBD, a homo- and/or a heterodimerization surface, and the ligand-dependent transactivation function AF-2 (for reviews see refs 1–5, 13 and therein). Within AF-2 of several nuclear receptors, an autonomous activating domain (AF-2 AD) has been recognized^{14–17}; its core integrity is required to support ligand-induced interaction with putative transcriptional intermediary factors (TIFs) which are believed to act as mediators between AF-2 and the transcriptional machinery (refs 18–23 and therein). To elucidate the structural basis of the multiple functions present in the LBD/region E and the nature of the molecular 'switch' induced by ligand binding, it is essential to solve the three-dimensional structures of apo- and holoreceptors. To this end we have previously elucidated the crystal structure of the human RXR- α apo-LBD²⁴. We have now established the structure of the T-RA-liganded human RAR- γ LBD, which not only shows that the RAR- γ and RXR- α LBD folds are closely related, but also suggests a mechanism by which a transcriptionally active receptor is generated upon ligand binding.

Structure determination and refinement

Crystals of the hexahistidine-tagged human RAR- γ D₃E (residues 178–423 of hRAR- γ ^{1,25}) grown in the presence of T-RA diffract to 2.0 Å (Table 1a). They belong to the tetragonal space

group $P4_12_12$, with one monomer in the asymmetric unit. After an attempt to obtain the crystal structure by molecular replacement using the RXR- α LBD as a model had failed, the phase problem was solved by isomorphous replacement. An initial 6-Å resolution single isomorphous replacement (SIR) map using a mercury derivative allowed the location of a set of five α -helices, which in turn permitted the tracing of the largest part of the polypeptidic chain into a 3.5-Å electron-density combined map (partial model). An almost complete model was then built into a 3-Å resolution map through several cycles of model building using O²⁶ and phase combination using Sigma-A (part of CCP4 (ref. 27)). Sequence assignment was based on the three mercury binding sites (C237, C335 and C338) of the *p*-chloromercuribenzyldisulphonate (PCMBDS) derivative. The model was refined against data between 8 and 2 Å by least-square minimization and simulated annealing with X-PLOR²⁸. The final model includes 238 amino acids (L182 to P419, 1870 non-hydrogen atoms), one ligand molecule (22 non-hydrogen atoms), and 119 water molecules. No clear electron density was obtained for the 4 carboxy-terminal and the 25 amino-terminal residues that encompass the histidine tag (21 residues). The agreement factor R_{cryst} is 0.210 for data between 8 and 2 Å; R_{free} ²⁹ is 0.313 using 10% of the reflection set. Individual *B*-factors were refined as isotropic and restrained. The refinement statistics are shown in Table 1a. A representative portion of the final electron-density map is shown in Fig. 1a. The stereochemistry of the model was better than the average according to PROCHECK³⁰, showing no Ramachandran plot outlier.

A common fold for hRAR- γ and hRXR- α LBDs

The human RAR- γ holo-LBD (topology, Fig. 1b; ribbon drawings, Fig. 1c) forms an antiparallel α -helical 'sandwich', a fold first described for the hRXR- α apo-LBD²⁴. Eleven α -helices (H1 to H12) are organized in a three-layer structure with H4, H5, H6, H8 and H9 sandwiched between H1 and H3 on one side, and H7, H10 and H11 on the other (Figs 1b, c and 2a); for comparison note that H2, present in hRXR- α but not in hRAR- γ , has been considered in the numbering scheme. As in RXR- α , a conserved kink at A266, corresponding to that at G304 in hRXR- α , separates H4 and H5. Two topologically conserved β -strands (s1 and s2) form a β -turn inserted between loop 1–3 (connecting H1 to H3) and H3.

RAR- γ and RXR- α LBDs were superposed using the LSQ options from O²⁶ (Fig. 2b). The r.m.s. deviation (r.m.s.d.) was 1.50 Å for 164 matched Ca's out of 238 (8.9 Å over all α -carbons). Six helices (H1, H4, H5 and H8–H10) match quite

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TABLE 1 Crystal data and mutant analysis

(a) Crystallographic analysis

Data collection				
Data set	Native 1	Native 2	PCMBs 1	PCMBs 2
X-ray source	Laboratory	LURE	Laboratory	LURE
Wavelength (Å)	1.54	0.90	1.54	0.90
Resolution (Å)	28.8–3.6	8.0–2.0	23.9–3.5	6.5–3.0
Unique reflections	3,634	17,193	2,981	5,172
Completeness (%)	97.9	94.1	74.0	92.5
Multiplicity	6.7	3.8	2.8	3.7
R_{sym} (I) (%)	4.9	9.7	6.1	6.4
Reagent concentration (mM)			0.5	0.1
Soaking time (h)			2	24
SIR analysis (15–3 Å)				
Number of sites			3	3
Phasing power † (c/a)			1.78/1.18	1.96/1.48
R_{cull} ‡ (c/a)			0.64/0.72	0.63/0.61
Anomalous data				6.5–3.0 Å
$R_{\text{cull anom}}$ §				0.85
Mean f.o.m. (c/a/o)	0.65/0.56/0.58			
Refinement (against 15,632 reflections between 8 and 2 Å with a cutoff of 3σ)				
R_{cryst} ¶ (%)	21.0			
R_{free} # (%)	31.3			
Non-hydrogen atoms				
Protein	1,870			
Ligand	22			
Water molecules	119			
R.m.s.d. on bond length (Å)	0.013			
R.m.s.d. on bond angles (°)	1.667			
Average B-factor ☆ (Å ²)				
Protein	20.6			
Ligand	20.9			
Water molecules	31.0			

(b) Ligand binding and transactivation abilities of RAR mutants

Mutation in RAR-γ	K_d (T-RA) (nM)	K_d (9C-RA) (nM)	EC ₅₀ (nM)
Wild-type	0.3	0.3	0.8
F230Y	32		7
K264A	0.5		NM
A397T	5.0		1,000
αT403 (γ405)	3.3**		
M408A	4.2	NM**	1,000
αP407A (γP409)	3.2**	14**	
αP408A (γP410)	1.8**	7.9**	
αL409A (γL411)	0.9**	2.7**	
αL410A (γL412)	1.6**	NM**	

* $R_{\text{sym}}(I) = \sum_{hkl} \sum_i |I_{hkl,i} - \langle I_{hkl} \rangle| / \sum_{hkl} \sum_i I_{hkl,i}$, where $\langle I_{hkl} \rangle$ is the average intensity of the multiple $I_{hkl,i}$ observations for symmetry-related reflections.

† Phasing power (centric (c), and acentric (a)) = $\langle F_H \rangle / \langle \epsilon \rangle$, where $\langle F_H \rangle$ is the root-mean-square (r.m.s.) calculated heavy-atom structure factor and $\langle \epsilon \rangle$ is the r.m.s. lack of closure error.

‡ R_{cull} (centric (c), and acentric (a)) = $\langle \epsilon \rangle / \langle \Delta_{\text{iso}} \rangle$, where $\langle \Delta_{\text{iso}} \rangle$ is the r.m.s. isomorphous difference.

§ $R_{\text{cull anom}} = \langle \epsilon \rangle / \langle \Delta_{\text{anom}} \rangle$, where $\langle \Delta_{\text{anom}} \rangle$ is the r.m.s. anomalous difference.

¶ F.o.m. (centric (c), acentric (a), and overall (o)): figure of merit.

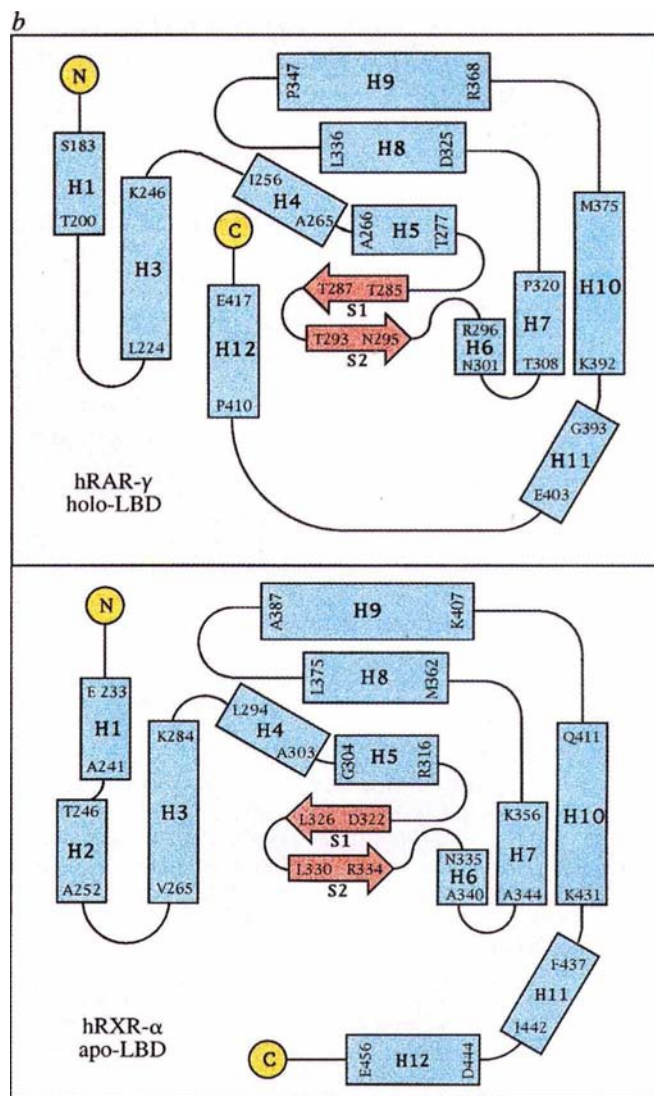
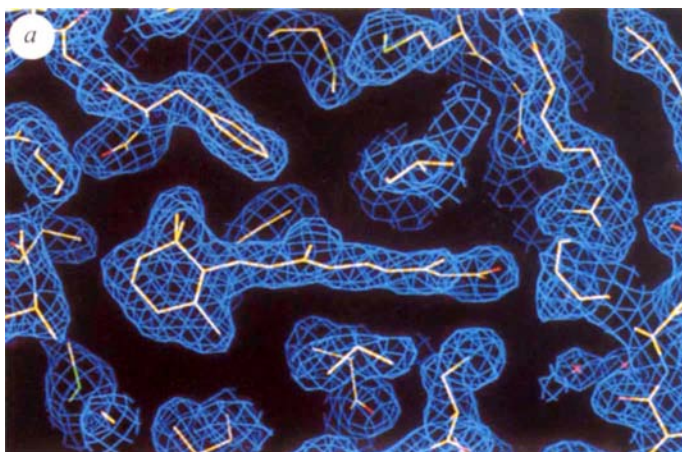
$R_{\text{cryst}} = \sum_{hkl} |F_{\text{obs}} - F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}|$.

$R_{\text{free}} = \sum_{hkl \in T} |F_{\text{obs}} - F_{\text{calc}}| / \sum_{hkl \in T} |F_{\text{obs}}|$, where the T set (10% of reflections) is omitted in the refinement²⁹.

☆ Average B-factor for non-hydrogen atoms.

** Data taken from ref. 36.

a, The RAR-γ D₃E (178–423)^{1,25} fused with a histidine tag (21 N-terminal amino acids) was overproduced using an *E. coli*/T7 system and purified by metal chelate affinity chromatography and gel filtration. Note that in contrast with RXR²⁴, hRAR-γ D₃E does not dimerize in solution, neither in the presence nor the absence of ligand (our unpublished data). Crystallization experiments were carried out with the hanging-drop vapour-diffusion method (5 μl protein solution, 5 μl precipitant solution and 0.5 ml reservoir) at 15 °C. Crystals of hRAR-γ D₃E grew in the presence of T-RA in 4 d from a solution of 0.75 M sodium acetate, 50 mM PIPES (pH 7.0), 250 mM NaCl, 5 mM Tris/HCl, 5 mM dithiothreitol, 1 mM CHAPS, 0.08 mM *n*-dodecyl-β-D-maltoside, and 2% glycerol equilibrated with a reservoir containing 1.5 M sodium acetate, 100 mM PIPES (pH 7.0). Crystals belong to the tetragonal space group P4₁2₁2, with $a = b = 60.6$ Å and $c = 155.3$ Å. Heavy-atom derivatives were obtained by soaking the crystals with a mercurial reagent (PCMBs 0.5 mM for 2 h or 0.1 mM for 24 h). X-ray data were collected at 0 °C with either a MarResearch imaging plate detector or a Siemens area detector mounted on a rotating-anode generator ($\lambda = 1.54$ Å) and at -5 °C on the W32 beamline of the LURE synchrotron (Laboratoire pour l'Utilisation du Rayonnement Electromagnétique, Orsay, France; $\lambda = 0.90$ Å). All computations were done on a DEC Alpha/OSF1 computer, and the graphic operations on an Evans & Sutherland station. Data were processed with the MarXDS package⁴⁰. Patterson and cross-Fourier analyses and SIR phasing were done using programs from the CCP4 package²⁷; building operations were carried out with O (ref. 26), and refinement with X-PLOR²⁸. Solvent molecules belong to the first shell of hydration and were located in difference Fourier maps. Individual B-factors were refined as



isotropic and restrained, and their values were in the range 15–40 Å². All solvent molecules have a temperature factor lower than 50 Å². Two alternative conformations were found for K236. In the first one, it makes a salt bridge with the T-RA carboxylate. In the second one, the side chain points towards the outside and makes a salt bridge with D351 of a symmetrical molecule; the former location of K236 N ϵ is occupied by a water molecule. Occupancy refinement indicates that the conformation toward T-RA represents ~40%. Both conformations can be seen in the density map by lowering the contouring level. The second conformation was considered a packing effect.

b, Dissociation constants of the various mutants were determined by Scatchard analysis by using crude extracts of the recombinant proteins expressed in *E. coli* according to Tate & Grippo³⁶. EC₅₀ values were derived from T-RA dose-response curves by using COS cells and (TRE)₃-tk-CAT (ref. 13) as reporter. αT403 corresponds to a mRAR-α truncated after P403 (ref. 14). NM, not measurable.

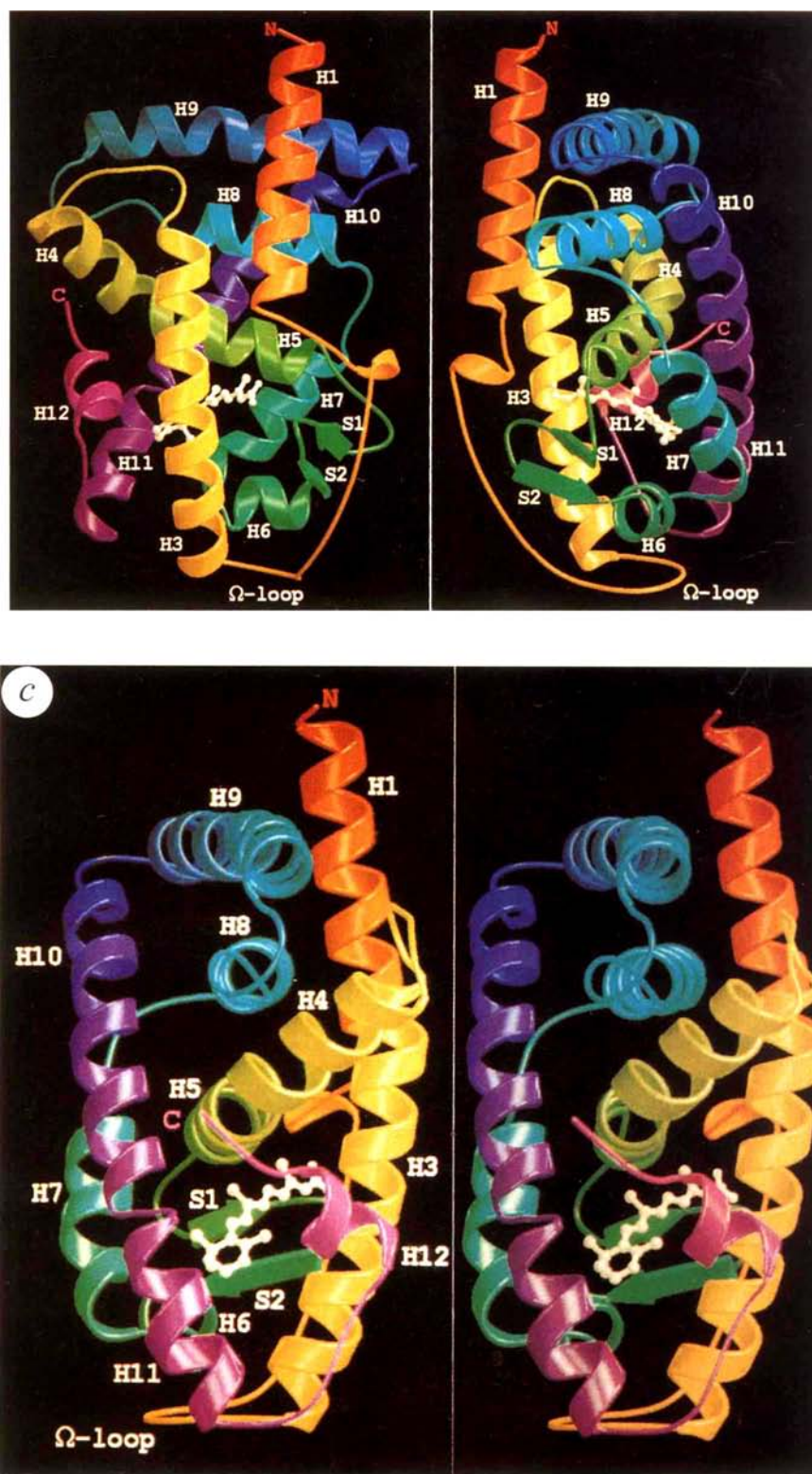


FIG. 1 a, View of the 2.0-Å resolution ($2F_o - F_c$) map contoured at 1.0 standard deviation, showing T-RA in its binding site. b, Topology diagram. The residues at the beginning and at the end of each α -helix or β -strand are indicated. For the sake of comparison with hRXR- α , helix 2 (H2), which does not exist in the hRAR- γ LBD structure, has been considered in the numbering scheme. c, Ribbon drawings of three views

(one stereoview at the top and two singles at $+150^\circ$ (left) and -100° (right) rotation) of the hRAR- γ LBD. The colour code is according to position in the sequence, starting with red at the N terminus (L182) towards purple at the C terminus (P419). T-RA is shown in white. Helices (H1, H3 to H12), β -strands (s1 and s2), and the Ω -loop are labelled.

well (r.m.s.d. values 0.99, 0.95, 1.02, 0.75, 1.15 and 1.12 Å, respectively). H7, which is shifted along the helical axis, shows an intermediary r.m.s.d. of 1.89 Å. H3, H6, H11 and the β -sheet show significantly larger deviations. The crystal structure comparison was used to align the RARs and RXRs over the

entire LBD sequence (Fig. 2a). This analysis shows that the breakpoint between H10 and H11 observed in hRXR- α (432-CLEHL-436) corresponds to a kink in the hRAR- γ extended H10-H11 helix (393-GA-394). Conversely, the disruption of the helical conformation into a loose linker peptide (405-PGPMP-

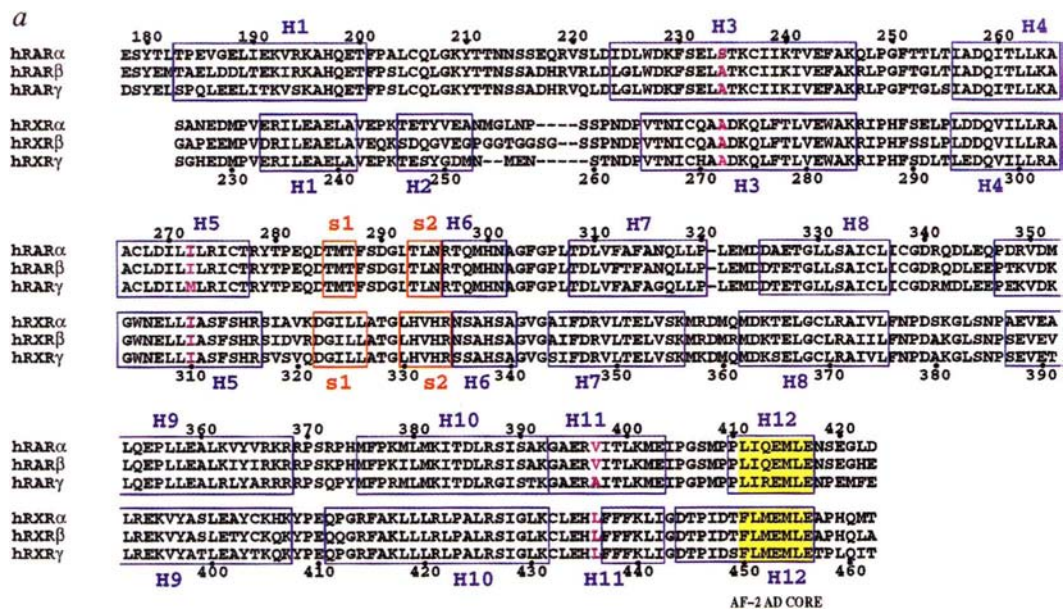
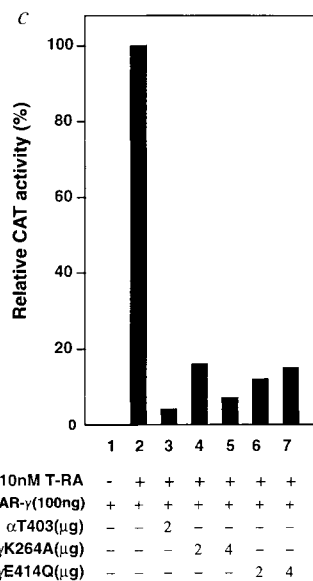
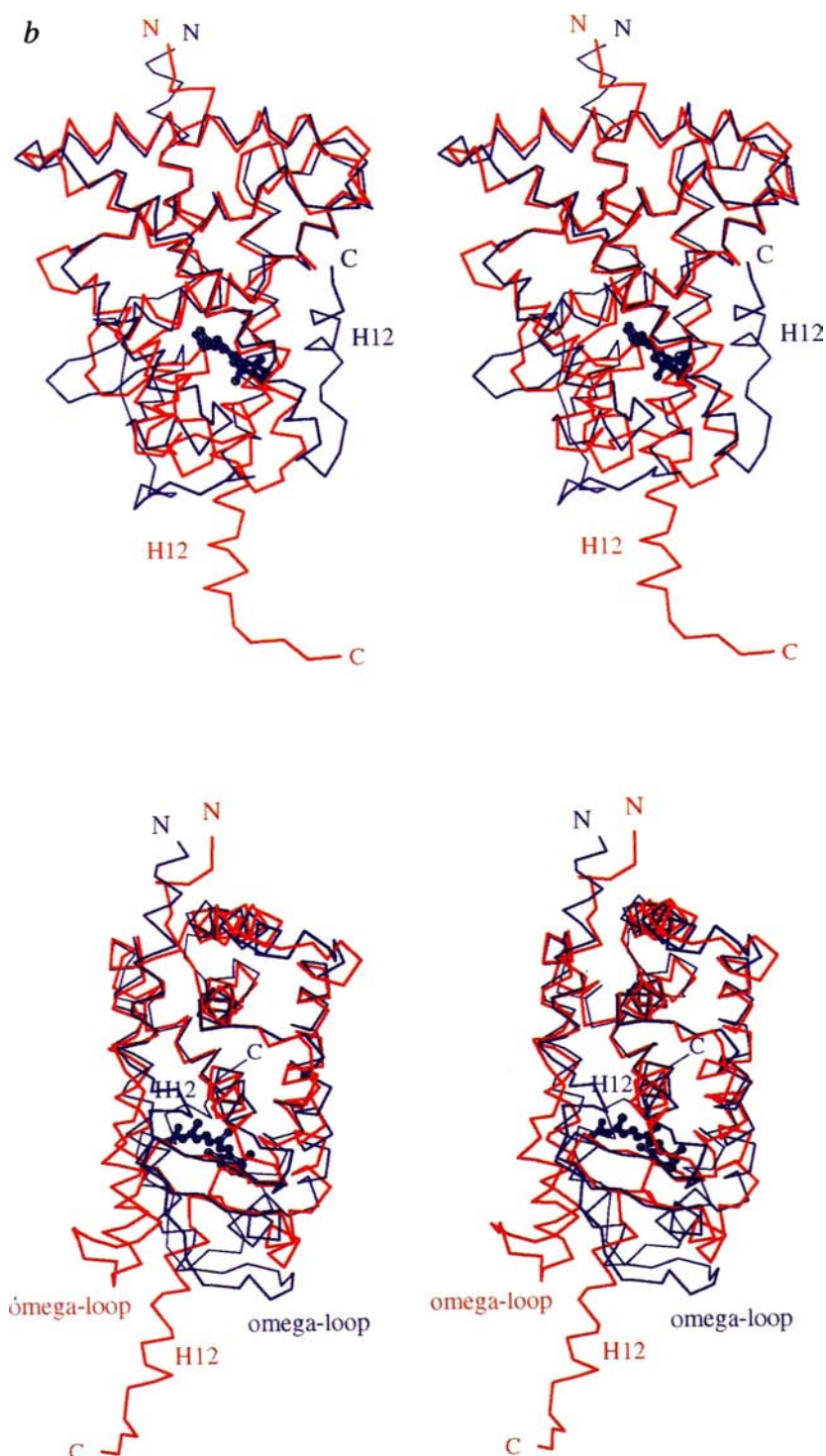


FIG. 2 a, Sequence alignment of LBDs of human RAR- α (refs 41, 42), β (ref. 43), γ (ref. 25) and human RXR- α (ref. 44), β (ref. 45), γ (J. M. Garnier and H.G., unpublished data) based on hRAR- γ and hRXR- α LBD crystal structure superposition. Amino-acid numbering is for hRAR- γ and hRXR- α . The secondary structure elements are boxed in blue (α -helices) or red (β -strands). The AF-2 AD core is highlighted in yellow. The three RAR- α , β or γ -specific residues in the ligand-binding pocket are indicated by purple letters. The two structures were superposed with LSQ in O (ref. 26). The r.m.s. deviation was 1.50 Å for 164 matched C α s. b, 115° rotated stereoviews of hRAR- γ /hRXR- α LBD backbone superposition. RAR is shown in blue, RXR in red and T-RA in a ball-and-stick fashion. The bottom stereoview has the opposite orientation relative to the stereoview of Fig. 1c. c, mRAR- γ K264A (see Table 1b) acts as dominant-negative mutant like α T403 (ref. 14) or RAR- γ E414A, in which the AF-2 AD core is lacking (α T403) or disabled (RAR- γ E414A; data not shown). COS cells were transfected with the indicated amounts of the various expression vectors together with the reporter gene (TRE) $_3$ -tk-CAT 13 and an internal control recombinant expressing β -galactosidase. CAT activities, normalized to equal units of β -galactosidase, are expressed relative to the T-RA/RAR- γ -induced CAT activity (100%). For a discussion of dominant-negative phenotypes see ref. 14.



409) connecting H11 to H12 in hRAR- γ corresponds to a marked kink in hRXR- α H11 (443-GDTP-446). Accordingly, we have now divided the previous H11 of RXR 24 into two separate helices, H11 and H12 (Figs 1b and 2a). Notably, the breakpoints between helices are conserved throughout the nuclear receptor superfamily 49 . Several structural differences account for the more compact structure of hRAR- γ holo-LBD when compared with hRXR- α apo-LBD. The connection between H1 and H3 (residues 201–223), which is constituted by an extended loop (201–211), interrupted by an α -helical-like segment (204–206) corresponding to H2 in RXR, and an Ω -loop (212–223), comes underneath the binding pocket following a different path. In RXR- α , the corresponding segment contains H2 and a similar Ω -loop oriented towards the opposite side of the protein core 24 (Figs 2b and 5). The interconversion between RAR and RXR conformations can be described as a hinged-lid motion of the Ω loop (first analysed in triose phosphate isomerase 31), concomitantly with an alternative orientation of H11 and H12. H12 protrudes from the LBD core in apo-RXR- α , whereas it folds back towards the LBD core in holo-RAR- γ . Note that the existence of a nearby solvent pocket in the crystal would allow H12 to protrude from the protein core. It is the position of the Ω -loop which prevents this

conformation. An essential electrostatic interaction involving N ζ of K264 (H4) with OE1 of E414 (2.91 Å), and to a lesser extent with OE1 of E417 (4.53 Å), stabilizes H12 in this position, along with a hydrogen bond between K264 N ζ and the carbonyl group of E414 (2.66 Å). The functional relevance of the K264–E414 salt bridge was tested by site-directed mutagenesis. The mutation K264A in hRAR- γ almost abolishes transactivation, without affecting ligand and DNA binding (Table 1b), or heterodimerization with RXR (not shown), thus resulting in a dominant-negative mutant (Fig. 2c). The only other intramolecular contacts of H12 and loop 11–12 involve the N-terminal part of H3, resulting in a kink at residue A234 (r.m.s.d. of 5.74 and 1.53 Å for residues 227–234 and 235–246, respectively). The bending of H3, which brings three residues of H3 (W227, F230 and A234) in contact with T-RA (see below), together with the $\sim 30^\circ$ tilt of H6 inwards (r.m.s.d. of 3.11 Å), contribute to a more compact structure. However, compared to RXR- α , the entire β -turn is shifted towards the surface (r.m.s.d. of 2.39 Å) and H11 is tilted outwards by $\sim 70^\circ$ (r.m.s.d. of 8.1 Å for residues 393–403). When the two LBDs are superposed, the carboxylate group and the β -ionone ring of T-RA in RAR- γ occupy the position of the β -turn and H11, respectively, in RXR- α (Fig. 2b).



In agreement with the absence of RAR LBD dimers in solution (ref. 13 and our unpublished data), the analysis of packing contacts does not reveal any potential dimers. The large dimeric interface involving H10, H9 and loop 7–8 through a two-fold crystallographic axis observed in RXR- α crystals²⁴ is not seen in the present structure.

The retinoic acid binding site

All-*trans* retinoic acid is buried in a predominantly hydrophobic pocket formed by residues located in H1, H3, H5, the β -turn, loop 6–7, H11, loop 11–12 and H12 (Fig. 3a, top). The hydrogen bonds and van der Waals contacts that stabilize the ligand are represented in Fig. 3b. This pocket essentially corresponds to

putative binding cavity A of RXR²⁴ with the β -ionone ring lying in putative cavity B, closed by a H11–H12 lid. T-RA is found in an extended all-*trans* conformation, slightly bent by the protein (Fig. 1a, c). H5 lies above the isoprene tail in an almost perpendicular orientation (Fig. 1c). In agreement with the crystal data, the mutations F230Y, A397T and M408A impair ligand binding and transactivation (Table 1b). A397 and M408 are located in the H11–H12 region, and residues belonging to the same region have been shown to be involved in T-RA binding by RAR- α (Table 1b). The carboxylate O21 makes a salt bridge with K236 N ζ (2.57 Å), itself hydrogen-bonded to the carbonyl group of L233 (2.60 Å), and is hydrogen-bonded to the main-chain amide group of S289 (2.71 Å). The carboxylate O22 inter-



FIG. 3 All-*trans* retinoic acid binding site. *a*, Stereoviews of T-RA (top) and modelled 9C-RA (bottom) in the hRAR- γ binding pocket. The protein C α trace is shown by a dotted line, a selection of contacting side chains by thick lines, and the ligand is represented in a ball-and-stick fashion. In the T-RA view, helix 12 side chains are shown. *b*, Schematic diagram of the van der Waals contacts (4.5 Å cut-off) and hydrogen bonds between the protein and the ligand, showing atom numbering for T-RA. When a residue makes contacts with several carbons of T-RA, only the shortest contacts are indicated. A solid box denotes a residue within 4.0 Å and a dotted box a residue within 4.5 Å. A helical wheel representation of H12 shows its amphipathic character and the hydrophobic residues that contact the β -ionone ring of T-RA. *c*, Schematic drawing showing the three pocket residues that are crucial for binding specificity between RAR- α , β and γ .

acts with the hydroxyl group of S289 (2.92 Å), which in turn is hydrogen bonded to R278 N η 1 (2.97 Å); R278 N η 2 also makes a weak salt bridge (3.17 Å) with the carboxylate O22. K220 and R269 of mouse RAR- β were predicted to interact with the carboxylate group of T-RA, because mutation of these residues to alanine reduces T-RA affinity³². Murine RAR- β R269 corresponds to hRAR- γ R278, which not only weakly interacts with the T-RA carboxylate, but also contributes to the hydrogen-bond network anchoring this group (Fig. 3*b*). However, mRAR- β K220 corresponds to hRAR- γ K229, which is not directly involved in the binding of the carboxylate, but may be involved in the electrostatic guidance of the ligand (see below).

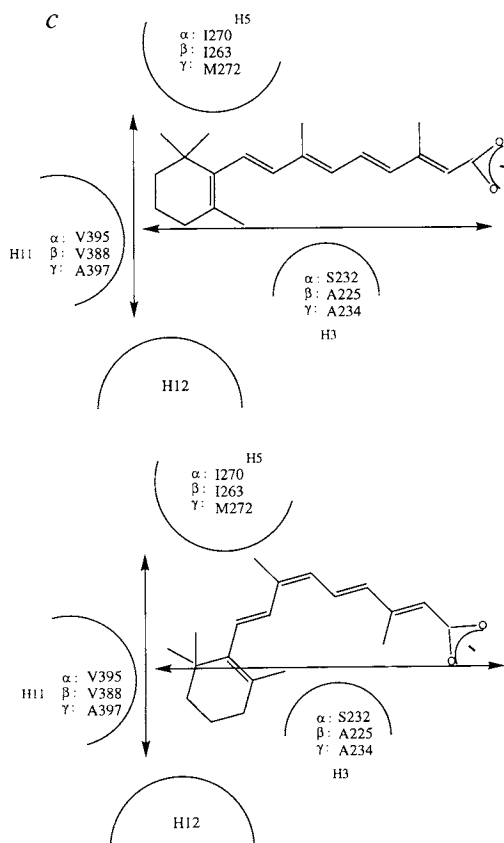
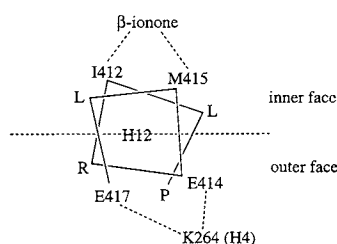
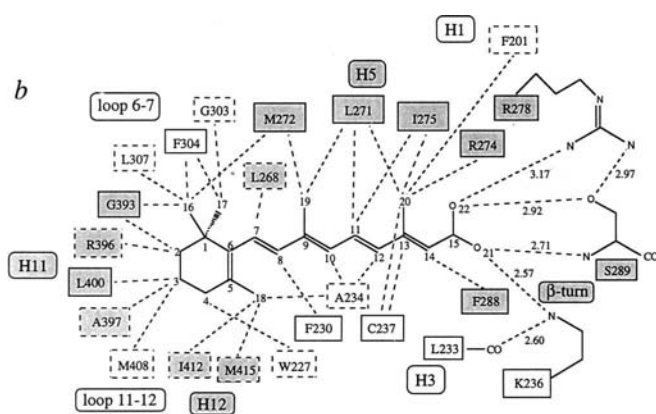
The T-RA binding site shows no similarity with those of the three known all-*trans* retinoic acid-binding proteins whose fold is a barrel composed of two orthogonal antiparallel β -sheets: rat epididymal retinoic acid binding protein (E-RABP)³³, bovine/murine cellular retinoic acid binding protein (CRABP) I (ref. 34) and human CRABP II (ref. 34). In CRABPs, T-RA exhibits an extended all-*trans* conformation,

but it adopts a 'pseudo-*cis*' conformation ('8-*cis*' retinoic acid) in E-RABP.

Ligand specificity of RARs

Sequence alignment of RAR- α , β and γ shows that only three residues in the ligand-binding pocket are variable: A234 (S232 in α and A225 in β), M272 (I270 in α and I263 in β), and A397 (V395 in α and V388 in β) (Fig. 3*c*). These divergent residues are obvious candidates to account for the RAR selectivity of certain synthetic retinoids (ref. 12 and therein). In RAR- γ , A234 and M272 interact with the isoprene tail, and A397 with the β -ionone ring. RAR- α and RAR- β differ only by one residue in the pocket (S232 (α) versus A225 (β) in H3). Indeed, the mutant RAR- α (S232A) acquired the ability to bind RAR- β -specific retinoids, as was reported for hRAR- α (S232A, T239I)³⁵ (P. Reczek, personal communication). Consideration of these three divergent residues should be useful for the design of RAR- α -, β - or γ -specific retinoids.

Because RARs bind both T-RA and 9C-RA, 9C-RA was modelled in the present RAR- γ LBD (Fig. 3*a*, bottom). Docking



of the ligand in the observed pocket is straightforward when applying the following criteria: a binding mode of the carboxylate group similar to that of T-RA, with the same anchoring amino acids (Fig. 3b); and no steric clash with the residues of the pocket. This second constraint imposes a slight shift of 9C-RA to accommodate F230 from helix H3. Energy minimization was performed from the most likely conformation. The β -ionone

ring of 9C-RA was found roughly superposed with that of T-RA, but closer to H11 and H12, making good van der Waals contacts, in keeping with the observation that C-terminal truncations and point mutations (M408A or I412A in RAR- γ numbering) in RAR- α abolish 9C-RA binding, but only reduce T-RA binding³⁶ (Table 1b). From the model, the lower affinity of 9C-RA for RAR- γ (compared with RAR- α and β ³⁷) could be explained by a steric hindrance between the C19 of 9C-RA and M272 in H5 which corresponds to an isoleucine residue in RAR- α and β (Fig. 3c).

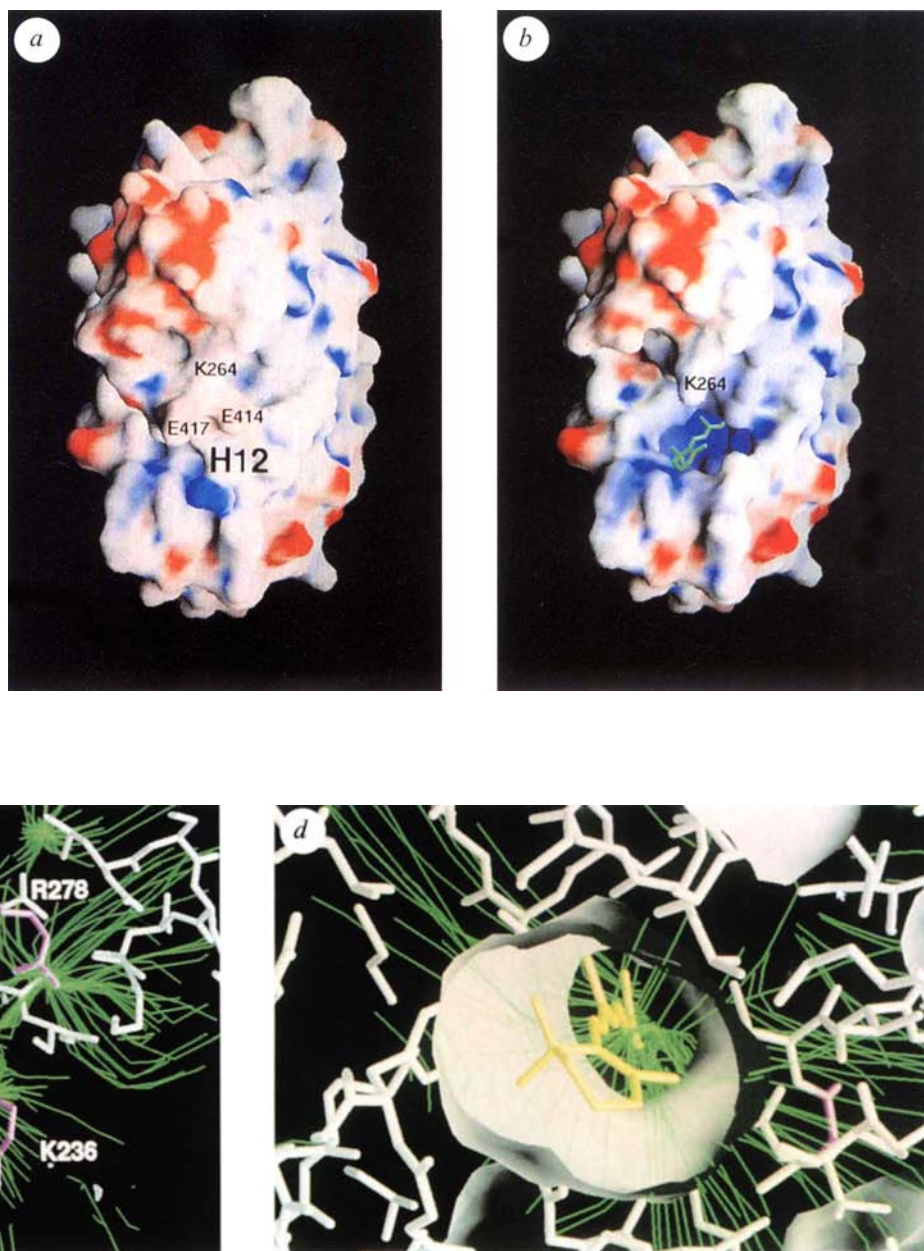
Electrostatic field guidance

Examination of the solvent-accessible surface of the molecule reveals no aperture to the ligand cavity (Fig. 4a). However, removal of the 10 C-terminal residues (H12) makes visible a large entry gate for which H12 could act as a 'lid' (Fig. 4b). In RXR- α , the corresponding entrance is only partly masked by the side chains of F438 and W305 (data not shown). Electrostatic field lines ending on the surface of the cavity are all oriented from negative to positive potentials and form a striking bundle leading to R278 and K236, which both anchor the T-RA carboxylate (Fig. 4c, d). Thus, provided that helix H12 is differently positioned in the absence of ligand, the carboxylate group is likely to enter the cavity first and be drawn down the hydrophobic gorge to its anchoring site, following the electrostatic field gradient. Entry from the opposite side of the cavity with the β -ionone ring ahead is unlikely because important local conformational changes would be required to allow the entry of the β -ionone ring, and because, when entering the cavity last, the carboxylate group would have to overcome the electrostatic barrier of the positively charged residues clustered at or near the surface in H3 and H5 (K229, K236, K240, R274 and R278). At the entrance of the cavity (Fig. 4b), K264 (H4) may help to attract T-RA from bulk solution and guide it to its binding site. Once T-RA is bound, K264, through salt bridges with E414 and E417, anchors H12 which seals the cavity (Fig. 4a).

LBD transconformation and transactivation

The hRAR- γ holo-LBD and hRXR- α apo-LBD structures are closely related, the major difference being the different positions of helix H12 (schematically illustrated in Fig. 5). It is therefore tempting to relate the two structures not to intrinsic differences between RAR and RXR, but to a ligand-induced transition that would convert a hRAR- γ apo-LBD structure similar to that of hRXR- α into the present hRAR- γ holo-LBD structure. Several lines of evidence support this hypothesis of two conformational states for apo- and holo-LBDs. First, although the entry site of the ligand binding pocket is closed by H12 in the hRAR- γ holo-LBD, it would be open in a structure modelled according to the hRXR- α apo-LBD. This would allow the ligand to enter into the ligand-binding cavity (Fig. 4a, b). Second, the conservation of the breakpoints at the end of H10 and between H11 and H12 in RAR and RXR is compatible with a homologous transition between the apo- and holo-LBD conformational states. Third, the T-RA and 9C-RA inducibility of transactivation by chimeric receptors in which RAR- α H12 is replaced by RXR- α or TR- α H12 actually demonstrates that helices H12 of different NRs are functionally interchangeable¹⁴. Fourth, in keeping with the hRAR- γ holo-LBD structure, RAR and RXR LBDs have been shown to be transformed upon ligand binding into a more compact structure that is less accessible to protease clipping (refs 38, 39 and therein). Finally, the putative NR transcriptional mediators, TIF1 and SUG1, bind to the holo- but not to the apo-LBDs of RAR and/or RXR^{18,22,23}, and these interactions require the presence of the AF-2 AD core, implying similar conformational transitions for the two receptors upon ligand binding. In this respect, it is striking that homologous mutations in helices H4 of mouse RAR- γ (K264A; corresponding to hRAR- γ K264A) and RXR- α (R307A; corresponding to

FIG. 4 Ligand accessibility and electrostatic attraction. *a*, The electrostatic surface potential of the hRAR- γ LBD, calculated with the program GRASP⁴⁶, is represented with the colour code red for negative and blue for positive potential. The view is the same as the stereoview in Fig. 1c, with H12 facing the reader. T-RA is present but not visible. *b*, Same view but with the C-terminal region (410–419) containing H12 omitted. The β -ionone end of the ligand (in green) is now visible at the entrance of a large cavity. *c*, *d*, Clipped views of the hRAR- γ LBD showing the ligand-binding pocket with electrostatic field lines (in green) ending on the surface of the cavity and oriented from negative to positive potentials. T-RA was not included in field line calculation with GRASP and was added (yellow). *c*, View approximately along H5 with H3 running at the bottom. Field lines divide into two branches ending on K236 and R278 (in magenta), respectively. *d*, View along T-RA. The van der Waals surface calculated for the LBD without the ligand and underlines the size of the cavity.



hRXR- α R302A) LBDs similarly decrease the interaction with TIF1 *in vitro* by approximately 80% (unpublished results). The RAR- γ H4 residue K264 is involved, through a salt bridge with the H12 residue E414 (and to a lesser extent with E417), in an essential electrostatic interaction which stabilizes H12 in its position in the RAR- γ holo-LBD. Homologous salt bridges may therefore exist in hRXR- α between the H4 residue R302 and the H12 residues E453 and E456 to stabilize H12 in a RAR- γ holo-LBD-like position allowing TIF1 to bind.

Previous studies have shown that the AF-2 AD core, which corresponds to the α -amphipathic helix H12, is an essential conserved element of the ligand-inducible activation function of nuclear receptors, including RARs and RXRs (refs 14–18, 23 and therein). Point mutations (for example, the mutation of mouse RAR- α residues E412 and E415 which correspond to hRAR- γ E414 and E417) within the AF-2 AD core generate dominant-negative mutants, which are impaired in transactivation, even though they are not affected in their ligand binding, DNA binding and dimerization capacities¹⁴. Strikingly, a mutant

with a similar dominant-negative phenotype is generated by mutation of H4 residue K264 (Fig. 2c; Table 1b), and the homologous mutation in hRXR- α (see above) also results in a mutant that is affected in transactivation, but not in ligand and DNA binding or dimerization (J. M. Wurtz *et al.*, manuscript in preparation). It appears, therefore, that the induction of the activation function AF-2 upon ligand binding corresponds to major conformational changes, particularly in the H11–H12 region, which create the proper surface required for efficient interaction with transcriptional factors, such as the putative mediators of the AF-2 function, TIF1, SUG1, ERAP160 or RIP140 (refs 18–23).

Conclusion

We currently favour a dynamic model in which the retinoid is attracted to the ligand-binding cavity by electrostatic forces and is locked in this position mainly through hydrophobic interactions. These interactions induce a structural transition which triggers a mechanism like a mouse trap (Fig. 5): pushed by the ligand, H11 is repositioned in the continuity of H10, and the

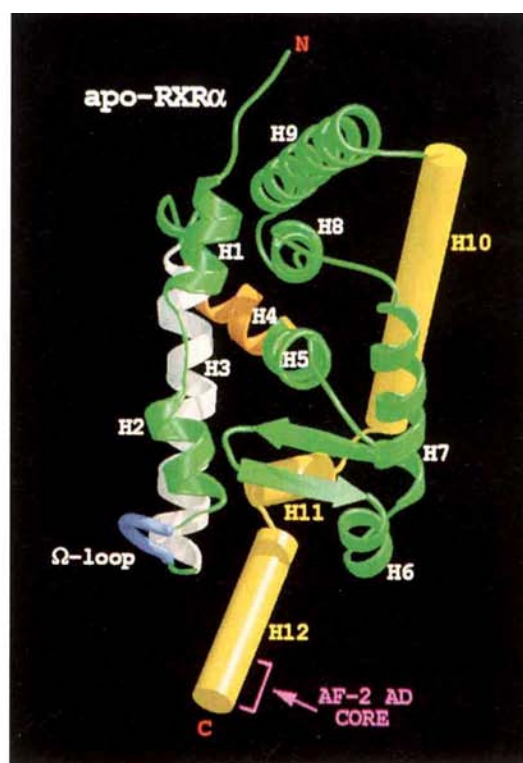
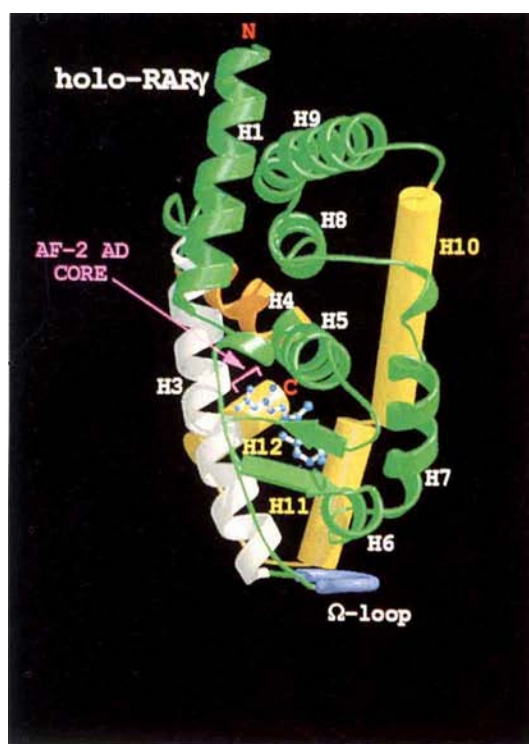


FIG. 5 Schematic representation of the hRXR- α apo-LBD (left) and hRAR- γ holo-LBD (right), emphasizing the large conformational changes between the two domains, in particular the swing of the C-terminal arm and the flip of the Ω -loop. Note also the bending of H3, which contributes to a more compact structure in RAR- γ . The orientation of the molecules is that of the bottom stereoview in Fig. 2b. H3 is shown in

white, H4 in orange, H10, H11, and H12 as yellow cylinders, and the Ω -loop as a thick blue coil. Helices extend as in Fig. 2a. T-RA is shown in a ball-and-stick fashion (with light blue atoms). MOLSCRIPT⁴⁷ was used to prepare Figs 1c, 2b, 3a and 5, and RASTER3D⁴⁸ to prepare Figs 1c and 5.

concomitant swinging of H12 unleashes the Ω -loop which flips over underneath H6, carrying along the N-terminal part of H3. In its final position, H12 seals as a 'lid' the ligand-binding cavity and further stabilizes ligand binding by contributing to the hydrophobic pocket. These conformational changes generate the surface which allows transcriptional mediators to bind. Confirmation of this model will require the elucidation of further crystal structures (matched apo- and holo-LBDs), refined mutagenesis experiments, and molecular dynamics modelling studies. □

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ACKNOWLEDGEMENTS. We thank J.-M. Wurtz for modelling 9C-RA in the RAR- γ LBD and for analysis of RAR specificity; C. Zecheil for *in vitro* interaction assays with TIF1 and SUG1; A. Litt, A. Mitschler, R. Ripp, D. Bonnier, C. Erb, J. M. Garnier, F. Granger, T. Lerouge and A. Poron for technical assistance; and J. P. Benoit, R. Fourme and the staff of LURE (Orsay) for help during data collection. This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique (including support from IMABIO), the Centre Hospitalier Universitaire Régional, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale, the Ministère de la Recherche et de la Technologie and Bristol-Myers-Squibb.

Received 26 September; accepted 31 October 1995.

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A structural role for hormone in the thyroid hormone receptor

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The crystal structure of the rat α_1 thyroid hormone receptor ligand-binding domain bound with a thyroid hormone agonist reveals that ligand is completely buried within the domain as part of the hydrophobic core. In addition, the carboxy-terminal activation domain forms an amphipathic helix, with its hydrophobic face constituting part of the hormone binding cavity. These observations suggest a structural role for ligand, in establishing the active conformation of the receptor, that is likely to underlie hormonal regulation of gene expression for the nuclear receptors.

THE nuclear receptors, members of a superfamily of eukaryotic transcription factors, regulate gene expression in response to binding small, hydrophobic ligands^{1–3}. The receptors exhibit a modular structure, with functionally separable domains. The most highly conserved domains are the DNA-binding domain (DBD) and the ligand-binding domain (LBD)^{3,4}. The DBD recognizes the specific DNA response element for the receptor^{2,3,5}. Several structures of receptor DBDs have been determined, and many principles governing DNA recognition have been established^{6–8}. The LBD participates in several activities, including hormone binding, homo- and/or heterodimerization, formation of the heat-shock-protein complex, and transcriptional activation and repression^{1,2}. Hormone binding induces changes in receptor conformation that control these properties and influence gene expression^{1,2}. Therefore, the three-dimensional structure of a liganded LBD is critical for understanding the structural mechanisms of hormone action.

Nuclear receptors for the thyroid hormones, of which 3,5,3'-triiodo-L-thyronine (T_3) is the most important in mammals, regulate the transcription of gene products that mediate hormone effects on differentiation, development and metabolism⁹. Two distinct genes, α and β , produce thyroid hormone receptors (TRs)⁹. Here we report the crystal structure of the LBD from the rat α_1 TR isoform (rTR α_1) with ligand bound at 2.2 Å resolution. The structure shows that hormone completes the hydrophobic core of the active conformation of the receptor. In addition, the C-terminal ligand-dependent activation domain contributes to hormone binding, allowing direct regulation by hormone. The recent publication of the crystal structure of the unliganded human α retinoid X receptor (hRXR- α), another nuclear receptor, provides an opportunity to consider differences between the two structures which may result from hormone binding¹⁰. These differences suggest mechanisms through which hormone regulates TR function. We expect the structural role for ligand revealed in this study, and the control which that role provides over key functional elements of the receptor, to be general for the entire class of nuclear receptors.

Structure determination and refinement

A fragment of the rat α_1 TR isoform (residues Met 122–Val 410), beginning immediately after the DBD and containing the entire LBD, was expressed in *Escherichia coli* using a T7 polymerase-based expression system¹¹ (Fig. 1). Selective purification of the bound rTR α_1 LBD was achieved through hydrophobic interaction chromatography of receptor saturated with ligand. Cocrystals of the rTR α_1 LBD, with either 3,5,3'-triiodothyronine

(T_3), 3,5-dibromo-3'-isopropylthyronine (IpBr $_2$), or 3,5-dimethyl-3'-isopropylthyronine (Dimit) prebound, were produced by vapour diffusion at ambient temperature from 15% 2-methyl-2,4-pentandiol (MPD) as previously described¹². The unique composition of the thyroid hormone allowed a phasing strategy in which cocrystals of the rTR α_1 LBD containing hormone analogues that differ at the 3,5 and 3' positions (T_3 , IpBr $_2$ and Dimit) provided isomorphous derivatives. In this set of analogues, the halogen substituents (2 Br and 3 I atoms) function as heavy atoms, while the Dimit cocrystal acts as the parent. The initial 2.5-Å multiple isomorphous replacement/anomalous scattering/density-modified electron-density map allowed the LBD to be traced from skeletons created in the molecular graphics program O¹³ (Fig. 2a). A model of the LBD was built in four fragments, Arg 157–Gly 182, Trp 185–Gly 197, Ser 199–Pro 205, and Val 210–Phe 405, and refined in XPLOR using positional refinement and simulated annealing protocols¹⁴. Missing residues were built with the aid of difference density, except His 184, Gln 198 and Gly 207, which could not be modelled. The final model was refined to $R_{\text{cryst}} = 21.8\%$ and $R_{\text{free}} = 24.4\%$ for data from 5.0 to 2.2 Å (Table 1; Fig. 2b).

Architecture of the TR LBD

The rTR α_1 LBD consists of a single structural domain packed in three layers, composed of 12 α -helices, H1–12, and four short β -strands, S1–4, forming a mixed β -sheet (Fig. 3a). The buried hormone and three antiparallel α -helices, H5–6, H9 and H10, form the central layer of the domain (Fig. 3b). H1, H2, H3, H4 and S1 form one face of the LBD, with the opposite face formed by H7, H8, H11 and H12. The predominantly helical composition and the layered arrangement of secondary structure is identical to that of the unliganded hRXR- α , confirming the existence of a common nuclear receptor fold suggested by sequence analysis⁴.

The first 35 amino acids of the N terminus (Met 122–Gln 156) are not visible in the electron-density maps. Amino acids corresponding to Met 122–Ile 151 of the rTR α DBD make extensive contacts with the minor groove of the DNA in the structure of the TR:RXR DBD heterodimer⁸. The five disordered amino acids (Arg 152–Gln 156), which reside between the last ordered residue of the TR DBD and the first ordered residue of the LBD, probably represent the effective 'hinge' that links the DBD and LBD in the intact receptor.

The TR LBD is visible beginning at Arg 157, and continues in an extended coil to the start of H1. A turn of α -helix, H2, covers the hormone-binding cavity. Immediately following, a

FIG. 1 Comparison of the sequences for the rat α_1 and human β thyroid hormone receptor ligand binding domains with the sequence for the human RXR- α LBD. The amino acid sequences of the α_1 TR LBD and hRXR- α LBD are 21% identical and 47% similar. The secondary structure elements observed for rat α_1 TR LBD and the hRXR- α are shown above the sequence: α -helices designated in blue, β -strands in yellow. Residues that contact the hormone are shown in purple type. The motif implicated in transcriptional activation, $\Phi\Phi\Phi\Phi$, where Φ represents a hydrophobic residue, is boxed.

			H1		H2	S1			
rTR α	156	QRPEPTPEEW	DLIHVATEAH	RSTNAQGSWH	KQRRKFLPDD	IGQSPIVSMF			
hTR β	210	HKPEPTDEEW	ELIKTVTEAH	VATNAQGSWH	KQRRKFLPED	IGQAPIVNAP			
hRXR α	225	SANEDMPVER	ILEAELAVEP	KTETTYVEANM	GLNPSSPND				
			H1		H2				
			H3		H4		H5-H6		
rTR α	206	DGDKVDLEAF	SEFTKIITPA	ITRVVDFAKK	LPMFCELPCE	DQIILLKGCC			
hTR β	260	EGGKVDLEAF	SHFTKIITPA	ITRVVDFAKK	LPMFCELPCE	DQIILLKGCC			
hRXR α	264	PV	TNICQAADKQ	LFTLVWAKR	IPHFSELPLD	DQVILLRAGW			
			H3		H4		H4-H5		
			H5-H6	S2	S3	S4	H7	H8	
rTR α	256	MEIMSLRAAV	RYDPESDTLT	LSGEMAVKRE	QLKNGGLGVV	SDAIF.ELGK			
hTR β	310	MEIMSLRAAV	RYDPESDTLT	LNGEMAVTRG	QLKNGGLGVV	SDAIF.DLGM			
hRXR α	306	NELLIASFSH	RSIAVKDGIL	LATGLHVHRN	SAHSAGVGAI	FDRVLTELVS			
			H4-H5	S1	S2	H6	H7		
			H9		H10				
rTR α	305	SLSAFNLDLT	EVALLQAVLL	MSTDRSGLLC	VDKIEKSQEA	YLLAFEHYVN			
hTR β	359	SLSSFNLDLT	EVALLQAVLL	MSSDRPGLAC	VERIEKYQDS	FLAFEHYIN			
hRXR α	356	KMRDMQMDKT	ELGCLRAIVL	FNPDSKGLSN	PAEVEALREK	VYASLEAYCK			
			H8		H9				
			H11		H12				
rTR α	355	HRKHNIPHFV	PKLLMKVTDL	RMIGACHASR	FLHMKVCEPT	ELFPPLFLEVF	EDQEV	410	
hTR β	409	YRKHHVTHFV	PKLLMKVTDL	RMIGACHASR	FLHMKVCEPT	ELFPPLFLEVF	ED	461	
hRXR α	406	HKYPEQPGRF	AKLLRLPAL	RSIGLKCLEH	LFFFKLIGDT	PIDTFLMEHLE	APHQMT	462	
			H10		H11				

short β -strand, S1, forms the edge of the mixed sheet, parallel to S4, the outermost of the three antiparallel strands. The chain is mostly irregular until H3 begins, antiparallel to H1. H3 bends at Ile 221 and Ile 222, which contact the ligand. The chain turns almost 90° at the end of H3 to form an incomplete α -helix, H4. This is followed by the first buried core helix, H5-6, its axis altered by a kink near the ligand at Gly 253. The helix is composed of mostly hydrophobic side chains, interrupted by two striking exceptions: Arg 262 is solvent inaccessible and interacts with the ligand carboxylate, and Glu 257 meets Arg 329 from H9 and Arg 375 from H11 in a polar invagination. H5-6 terminates in a short β -strand, S2, of the four-strand mixed sheet. S3 and S4 are joined through a left-handed turn, and further linked by a salt bridge between Lys 283 and Asp 272. Following S4, H7 and H8 form an L, stabilized by a salt bridge between Lys 288 and Asp 297. The turn between H7 and H8 adopts an unusual conformation, a result of interaction with ligand and its glycine-rich sequence. The second core helix, H9, is antiparallel to the adjacent H5-6, and contains two buried polar side chains, Glu 315 and Gln 320. Glu 315 forms a buried salt bridge with Arg 356 and His 358. The oxygen of Gln 320 forms a hydrogen bond with the buried side chain of His 175. The chain then switches back again to form H10, also antiparallel to H9. H11 extends diagonally across the full length of the molecule. Immediately after H11, the chain forms a type II turn, at approximately 90° to H11. The chain then turns again to form H12, which packs loosely against H3 and H11 as part of the hormone-binding cavity. The five C-terminal amino acids, Glu 406–Val 410, are disordered.

The hormone-binding cavity

Dimit, an isostere of T_3 , is a thyroid hormone agonist¹⁵. Therefore, the conformation of the Dimit-bound receptor should resemble the T_3 -bound conformation. The ligand is buried within the receptor, providing the hydrophobic core for a sub-domain of the protein (Fig. 3b, c); H5-6 and H9 complete the bipartite core of the receptor. An extensive binding cavity is constructed from several structural elements. The cavity is enclosed from above by H5-6 (Met 256–Arg 266), from below by H7 and H8 and the intervening loop (Leu 287–Ile 299), and along the sides by H2 (Trp 185–Gln 187), by the turn between S3 and S4 (Leu 276–Ser 277), by H3 (Phe 215–Arg 228), by H11 (His 381–Met 388) and by H12 (Phe 401–Phe 405). The volume of the cavity, calculated by GRASP (600 Å³), is essentially the volume of the hormone (530 Å³). The remaining volume is occupied by water molecules surrounding the amino-propionic acid substituent. The contacts between the LBD and the ligand are shown in Fig. 4a, b.

The inner and outer rings of the ligand are nearly perpendicular, with the 3' substituent of the outer ring projecting down and

away from the inner ring, as predicted by studies of receptor binding to conformationally restricted analogues¹⁵. The amino-propionic acid and the outer phenolic ring assume the transoid conformation, each on opposite sides of the inner ring. The torsion angle χ_1 for the amino propionic acid is 300°.

The amino-propionic acid substituent is packed loosely in a polar pocket formed by side chains from H2, H4 and S3. The carboxylate group forms direct hydrogen bonds with the guanidinium group of Arg 228 and the amino nitrogen of Ser 277. In addition, Arg 262, Arg 266 and Asn 179 interact with the carboxylate through water-mediated hydrogen bonds. No hydrogen bond is formed by the amino nitrogen. The interactions of the amino-propionic acid substituent are consistent with 3,5-diiodo-3'-isopropylthyroacetic acid, or triac, which lacks the amino nitrogen, having a binding affinity equal to that of T_3 , suggesting that the amino nitrogen and longer aliphatic chain of T_3 do not contribute greatly to binding affinity¹¹.

The diphenyl ether, in contrast, is buried within the hydrophobic core. The inner ring packs in a hydrophobic pocket formed by H3, H5-6 and S3. Pockets for the 3- and 5-methyl substituents are not completely filled, as expected because the van der Waals radius of the methyl substituent of Dimit is smaller than that of the iodine substituent provided by the thyroid hormone T_3 .

The outer ring is packed tightly in a pocket formed by H3, H5-6, H7, H8, H11 and H12, and the loop between H7 and H8. The ether oxygen is in a hydrophobic environment defined by Phe 218, Leu 276, Leu 287 and Leu 292. The absence of a hydrogen bond to the ether oxygen is consistent with its proposed role in establishing the correct stereochemistry of the phenyl rings, as suggested by potent binding of hormone analogues with structurally similar linkages that possess reduced or negligible hydrogen bonding capability¹⁵. The 3'-isopropyl substituent contacts Gly 290 and Gly 291. The structure of the pocket may explain why activity is highest for a 3'-isopropyl substituent, and decreases with added bulk¹⁵. The only hydrogen bond in the hydrophobic cavity is formed between the phenolic hydroxyl and His 381 N ϵ 2. The conformation of His 381 is stabilized by packing contacts provided by Phe 405, and Met 256.

The presence of a 5' substituent larger than hydrogen decreases the binding affinity of the hormone. The more abundant thyroid hormone, 3,5,3',5'-tetraiodo-L-thyronine (T_4), contains an iodine at this position, and binds the receptor with 2% of the affinity of T_3 . The structure suggests that discrimination against T_4 is accomplished through the combination of steric conflict by Met 256 and possibly the constraints imposed by the geometry of the hydrogen bond from His 381 to the phenolic hydroxyl.

Deletion and antibody competition studies suggest the involvement of residues Pro 162 to Val 202 in ligand binding^{16,17}.

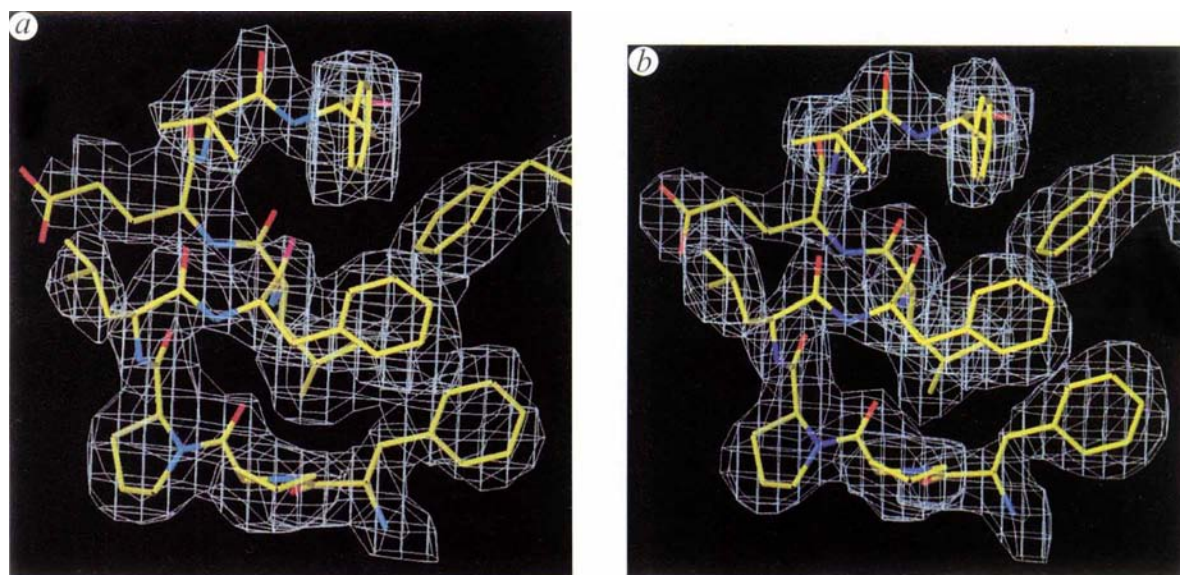


FIG. 2 *a*, The experimental, MIRAS/density modified electron density at 2.5 Å, contoured at 1σ . The depicted region is the C-terminal activation domain, residues Pro 399–Phe 405. The refined model, shown as a

stick representation, is superimposed. *b*, The corresponding refined $2F_o - F_c$ electron density at 2.2 Å, contoured at 1σ .

The region does not directly contact hormone in the bound structure, although H2 packs against residues forming the polar pocket that interacts with the amino-propionic acid group. Thus H2 may stabilize these residues in the bound state. H2, with β -strands S3 and S4, might also represent a prevalent entry point for ligand. Studies of receptor binding to T_3 affinity matrices demonstrate that only a linkage to the amino-propionic acid is tolerated, suggesting that steric hindrance present in other linkages prevent binding¹⁸. Furthermore, the crystallographic temperature factors suggest the coil and β -strand region is the most flexible part of the domain (data not shown). Participation of this region, part of the hinge domain between the DBD and LBD, in binding hormone may provide structural means for ligand binding to influence DNA binding, as parts of the hinge domain contact DNA^{2,8}.

Generalized resistance to thyroid hormone

The syndrome generalized resistance to thyroid hormone (GRTH) is characterized by high serum levels of T_3 and T_4 with non-suppressed levels of thyroid stimulating hormone (TSH), and a generally euthyroid state¹⁹. Genes from these patients contain mutations in the $TR\beta_1$ LBD, clustered within two regions in the linear sequence. The mutant receptors exhibit reduced to undetectable hormone binding activity, and typically antagonize the activity of wild-type receptors. The antagonism can be overcome in many cases by elevated concentrations of hormone which compensate for the mutant receptors' reduced affinity. Because the $rTR\alpha_1$ LBD shares 88% sequence identity with the $hTR\beta_1$ LBD, the bound structure can serve as a model for study of the GRTH mutations.

GRTH mutations cluster into two regions of secondary structure: S4, H6, H7 and the connecting loop with H8 (cluster 1); and H11 and H12 (cluster 2). Provocatively, the mutations describe the subdomain of the receptor for which hormone provides the hydrophobic core (Fig. 4c). The apparent pattern confirms the central effect of GRTH on hormone binding and hormone-dependent processes. The mutations appear to influence ligand binding by either disrupting direct contacts, or altering interactions that stabilize the bound conformation. Furthermore, the mutations would be expected to vary in their quantitative effect on ligand binding, and also in the ability of ligand to convert the mutated receptor to the active conformation. Moreover, receptor activities retained by the mutant recep-

tors, such as dimerization, localize outside the GRTH cluster, and are probably unaffected^{20,21}.

Dimerization and heterodimerization

The TR is capable of both homodimerization, and heterodimerization with RXR, on appropriate DNA response elements^{1,5}. Several mutations which affect TR dimerization involve a heptad repeat of hydrophobic residues (Leu 367–Leu 374) that is proposed to form a protein interface similar to a leucine zipper^{20,22}.

The $rTR\alpha_1$ LBD constructs the crystal lattice as a monomer. The limited surface area buried by the intermolecular contacts between monomers suggests the contacts do not represent a physiological dimer association. The heptad repeat is located at the N-terminal portion of H11. Many of the residues implicated in dimerization are found on the surface of H11, forming an extensive hydrophobic patch. The face of the $rTR\alpha_1$ LBD formed by H10 and H11 is composed mainly of hydrophobic residues, with alternating concave and convex surface, and appears suited to protein interaction. In the $hRXR-\alpha$, the N-terminal portion of H10, together with H8 and H9, does indeed form a dimerization surface¹⁰.

Transcriptional activation domains

The C-terminal 17 amino acids of the TR, referred to as the C-terminal activation domain, are implicated in hormone-dependent transcriptional activation. Mutation of key residues within the domain decrease or abolish ligand-dependent activation^{23,27}. Furthermore, the domain functions autonomously as a transcriptional activator when fused to an unrelated DBD^{23,24,27}. Thus, the region may represent a discrete functional domain, which in the receptor is regulated by hormone. Deletions or mutations of this domain also affect affinity for hormone, further evidence that ligand binding and activation are linked. Similar activation domains have been identified in the other nuclear receptors^{28,30}. The domain is proposed to form an amphipathic helix, within which the highly conserved motif $\Phi\Phi\Phi\Phi\Phi$ (where Φ represents a hydrophobic residue) is proposed to mediate interactions between the receptors and transcriptional coactivators^{23,26}. Recently, several proteins were identified which bind the TR, or other nuclear receptors, in a ligand-dependent manner through the C-terminal domain^{31,34}. (D. D. Moore, personal communication).

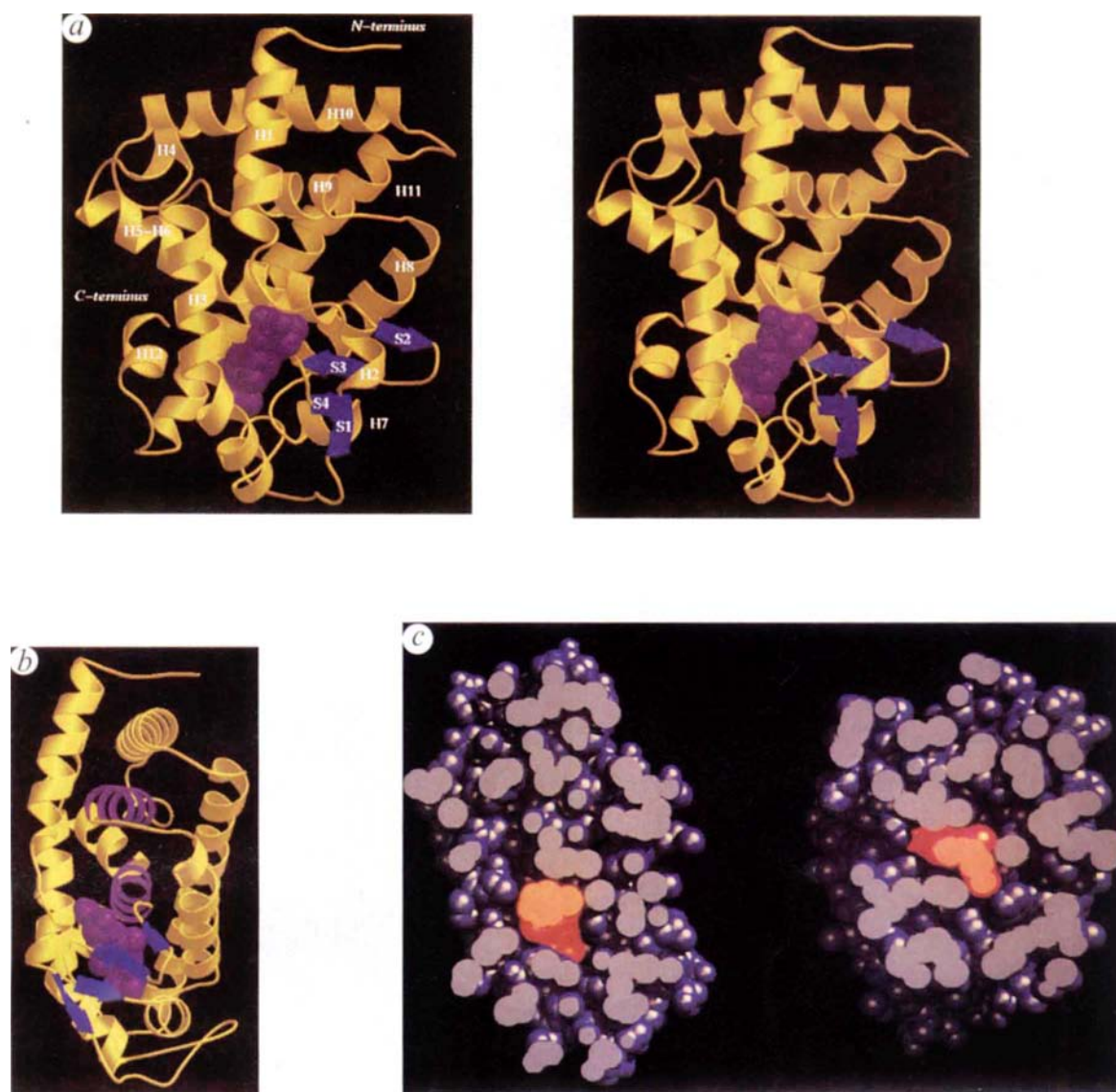


FIG. 3 *a*, Stereo drawing of the $rTR\alpha_1$ LBD, with secondary structure elements labelled. The hormone (magenta) is depicted as a space-filling model; α -helices and coil conformations are yellow, β -strands are blue. Secondary structure assignments were obtained using the Kabsch and Sander algorithm as implemented in PROCHECK⁴⁶. *b*, Ribbon drawing of the $rTR\alpha_1$ LBD, showing the three layers of the domain. The hydrophobic core of the receptor is formed by two α -helices and the hormone.

The hormone is depicted as a space-filling model. Core-forming elements are shown in magenta. *c*, The hormone is completely buried within the receptor. Two cross-sections of a space-filling model of the $rTR\alpha_1$ expose the hormone (magenta) tightly packed within the receptor. The left panel is a cross-section of the view in Fig. 3*b*; the right panel is a cross-section of a view rotated 90° about the horizontal from that in Fig. 3*b*.

The C-terminal activation domain of the TR forms an amphipathic helix, H12, which nestles loosely against the receptor to form part of the hormone binding cavity. The helix packs with the hydrophobic residues facing inward towards the hormone binding cavity, and the charged residues, including the highly conserved glutamate, extending into the solvent (Fig. 5*a*). Phe 401 interacts directly with the hormone, forming a van der Waals contact with the plane of the outer phenyl ring. Phe 405 interacts with His 381, perhaps stabilizing its hydrogen bonding conformation. Participation of Phe 401 and Phe 405 in binding hormone explains how mutation of these residues decreases hormone binding affinity²³. Furthermore, the impact of the these mutations on activation probably derives from a role in stabilizing the domain in the bound structure. Glu 403 extends into the solvent as an ordered residue, consistent with its critical role in

transactivation and presumed interactions with activator proteins (Fig. 5*b*). The other charged residues, Glu 406 and Asp 407 are disordered.

Two other sequences in the TR, $\tau 2$ and $\tau 3$, activate transcription when expressed as fusion proteins with a DNA-binding domain²⁴. The sequences, discovered in the $TR\beta$, correspond to $TR\alpha$ residues Pro 158–Ile 168 in H1 ($\tau 2$), and Gly 290–Leu 319 in H8 and H9 ($\tau 3$). Unlike the C-terminal activation domain, $\tau 2$ and $\tau 3$ do not appear to represent modular structural units in the $rTR\alpha_1$ LBD, nor present a surface for protein–protein interactions: the critical aspartate/glutamate residues of $\tau 3$ are located on two separate helices, and do not form a single surface; the charged residues of $\tau 2$ are engaged in ion pair interactions within the LBD. Thus $\tau 2$ and $\tau 3$ may not function as activation domains in the context of the entire receptor.

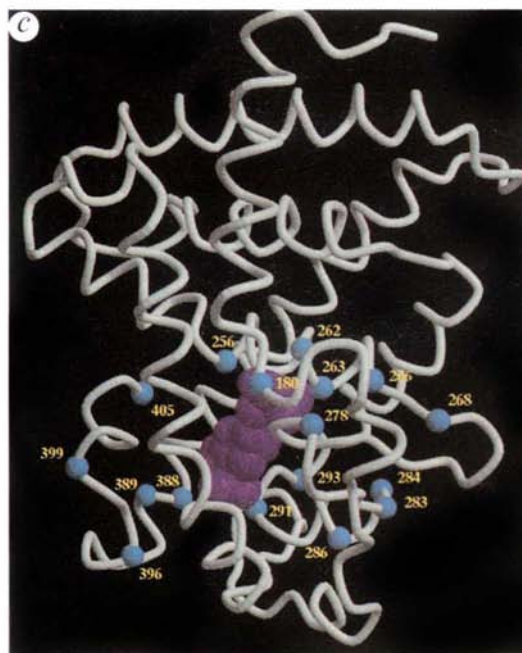
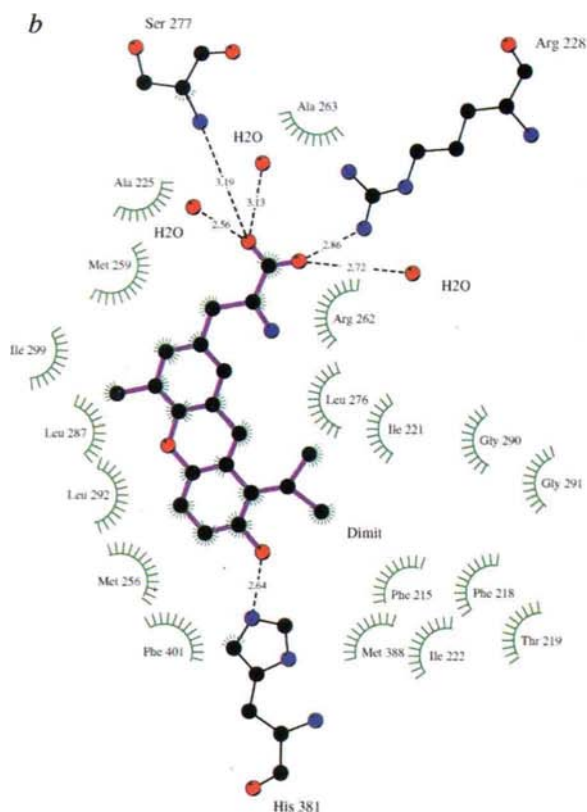
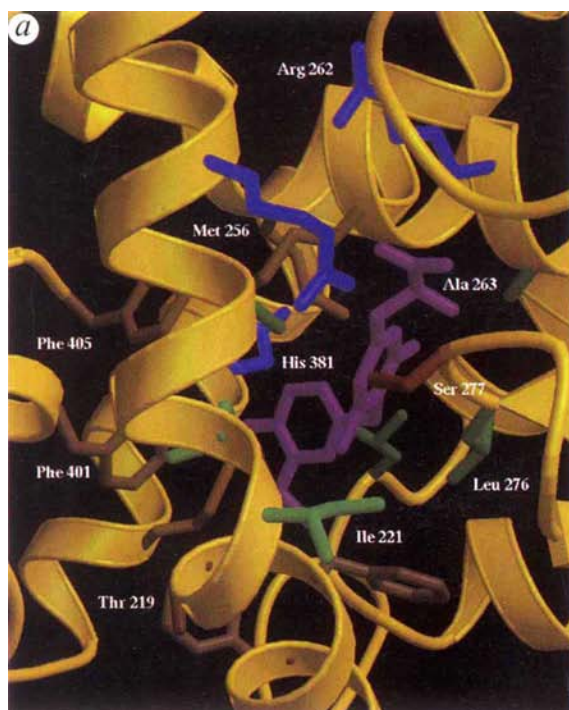


FIG. 4 a, Stereo view of the ligand binding cavity. Residues which interact with the ligand are shown as side chains. The colouring scheme reflects the chemistry of the side chain: basic residues (arginine and histidine) are blue, aromatic hydrophobics (phenylalanine) are brown, aliphatic hydrophobics (alanine and leucine) or light green (isoleucine), hydrophilic residues (serine and threonine) are dark red, and sulphur-containing residues (methionine) are dark gold. The ligand is coloured magenta. The view is identical to that of Fig. 3a. The residues are (clockwise from top): Arg 262, Ala 263, Ser 277, Leu 292 (left), Leu 276 (right), Ile 221, Phe 218, Phe 215, Thr 219, Met 388, Ile 222, Phe 401, Phe 405, His 381, Ala 225, Met 256 (left), Met 259 (right), Arg 228. b, Schematic diagram of the hormone binding pocket. Residues that interact with hormone appear at approximately the site of interaction. Hydrogen bonds are shown as broken lines between the bonding partners; distances for each bond are listed. Non-bonded

contacts are shown as radial spokes, which face towards interacting atoms. Only three direct hydrogen bonds are made: from Arg 228 and Ser 277 to the carboxylate group, and from His 381 to phenolic hydroxyl. The ether oxygen is found in a hydrophobic environment, surrounded by Phe 218, Leu 276, Leu 287 and Leu 292. Contacts to the inner ring are provided by the following side chains: Ile 221, Ile 222 and Ala 225 from H3; Met 259, Ser 260, Arg 262 and Ala 263 from H5; and Leu 276 from the loop between S3 and S4. Ser 260 and Ile 299; and Phe 218, Ile 221 and Ile 222 form pockets for the 3- and 5-methyl substituents, respectively. Contacts to the outer ring are provided by three phenylalanines in plane-perpendicular fashion: Phe 215, Phe 218 and Phe 401. Other side chain contacts are made by Gly 290–291 and Leu 292 from the loop between H7 and H8, His 381 and Met 388 from H11, and Ile 222 and Thr 219 from H3. Gly 290 and Gly 291 form a pocket for the 3'-isopropyl substituent. A subset of these interactions are described in the text. This figure was prepared using Ligplot 1.4.7. c, A selection of mutations observed in patients with GRTH mapped onto the rTR α 1 LBD⁴⁸. The positions of the residues are shown as blue spheres. The mutated residues are: A180T, M256T, R262H, A263T, R267C, D268H, G278R, A283T, R284W, Q286H, G291R, G293E, R384H, M388V, K389E, L396H, P399T and F405C.

TABLE 1 Data collection, phasing and refinement statistics

	DIMIT	rTR α_1 LBD with T $_3$	lpBr $_2$
Data Collection			
Cell dimensions			
a (Å)	117.16	117.19	117.18
b (Å)	80.52	80.20	80.12
c (Å)	63.21	63.23	63.13
β (°)	120.58	120.60	120.69
Resolution (Å)	2.2	2.0	2.1
Reflections	57031	64424	66877
Unique reflections	22327	21023	23966
Completeness (%)	87.0	82.4	93.7
R_{sym} (%) [*]	3.9	3.5	4.5
Phasing (15.0–2.5 Å)			
R_{der} (%) [†]		19.6	11.6
No. of sites		3	2
Occupancy [‡]		44.6 (19.8)	35.0
(Anomalous)		50.2 (23.7)	35.0
		39.2 (22.3)	
F_o/E_s			
Centric (acentric)			
15.0–5.0 Å		3.67 (4.61)	2.25 (3.09)
5.0–3.0 Å		2.23 (2.75)	1.25 (1.85)
3.0–2.5 Å		1.64 (1.99)	1.15 (1.57)
R_{cullis} (%)			
15.0–5.0 Å		33	44
5.0–3.0 Å		45	63
3.0–2.5 Å		60	65
Mean figure of merit	0.62		
Refinement (5.0–2.2 Å)			
R_{cryst} (%) [*]	21.8		
R_{free} (%)	24.4		
R.m.s. bond length (Å)	0.006		
R.m.s. bond angle (°)	1.245		

Rat α_1 TR LBD, residues Met 122–Val 410, was purified from *E. coli* as described¹¹. Material from a single preparation was divided into batches, and quantitatively bound with either Dimit, T $_3$ or lpBr $_2$ for the final purification step. Diffraction quality crystals (dimension $0.5 \times 0.2 \times 0.075$ mm³) were grown at ambient temperature from hanging drops containing 15% 2-methyl-2,4-pentane-diol (MPD) and 8.7 (Dimit), 5.5 (lpBr $_2$), or 2.3 (T $_3$) mg ml⁻¹ of protein as described¹². The crystals are of the monoclinic space group C2, with one monomer in the asymmetric unit. **Data collection.** Data from each cocrystal were measured on a Mar area detector at Stanford Synchrotron Radiation Laboratory beamline 7–1 ($\lambda = 1.08$ Å) using 1.2° oscillations; data from the T $_3$ cocrystal were measured with the b axis approximately parallel with the spindle. The crystals were flash frozen at –178 °C in a nitrogen gas stream with the MPD mother liquor serving as the cryosolvent. An orientation matrix for each crystal was determined using REFI⁴². Reflections were integrated with DENZO, and scaled with SCALEPACK (Z. Otwinowski). For the T $_3$ data set, Bijvoet pairs were kept separate, and locally scaled using MADSYS (W. Hendrickson and W. Weiss). **Phasing.** Cocrystals prepared from the three isosteric ligands were isomorphous. MIR analysis was performed using programs from the CCP4 suite⁴³. Difference Pattersons were calculated for both T $_3$ and lpBr $_2$, taking the Dimit cocrystal as the parent. The positions of the three iodine atoms in the T $_3$ difference Patterson were unambiguously determined from the Harker section of the density map as peaks of 11 σ above background. The positions for the two bromine atoms in the lpBr $_2$ were located independently, as peaks 8 σ above the noise level. Phases for the rTR α_1 LBD were calculated from the solution to the lpBr $_2$ difference Patterson, and used to confirm the location of the unique third iodine of the T $_3$ cocrystal. Halogen positions were refined with MLPHARE including the anomalous contributions from the iodine atoms⁴⁴. The MIRAS phases were improved through solvent flattening/histogram matching using DM⁴⁵. **Model building and refinement.** A model of the rTR α_1 LBD with Dimit bound was built with the program O from the solvent flattened MIRAS 2.5-Å electron-density map¹³. The initial model, without ligand ($R_{\text{cryst}} = 40.1\%$), was refined using least-squares protocols with XPLOR. The Dimit ligand was built into unambiguous $F_o - F_c$ difference density during the following round. Subsequent refinement used both least-squares and simulated annealing protocols with XPLOR¹⁴. Individual atomic B-factors were refined isotropically. As defined in PROCHECK, all residues are in allowed main-chain torsion angle regions⁴⁶. The current model is missing 35 residues (Met 122–Gln 156) at the N terminus, 5 residues (Glu 406–Val 410) at the C terminus, and the individual residues His 184, Gln 198 and Gly 207. In addition, the following residues were not modelled beyond C β due to poor density: 186, 190, 206, 209, 240, 302, 330, 337, 340, 343, 359, 395. The average B-value for protein atoms is 34.5 Å². The final model consists of the rTR α_1 LBD, residues Arg 157–Ser 183, Trp 185–Gly 197, Ser 199–Asp 206, and Asp 208–Phe 405; three acetylate-modified cysteines: Cys 334, Cys 380 and Cys 392; and 73 solvent molecules modelled as water (2,003 atoms). Coordinates will be deposited with the Brookhaven Protein Databank.

$$^* R_{\text{sym}} = 100 \sum_{hkl} \sum_i |I_i - \bar{I}| / \sum_{hkl} \sum_i I_i$$

$$^{\dagger} R_{\text{der}} = 100 \sum_{hkl} |F_{\text{PH}} - F_{\text{H}}| / \sum_{hkl} |F_{\text{H}}|$$

[‡] The occupancy for the two bromine sites is set to 35 electrons. The occupancies of the iodine sites are relative to this value.

Hormone-induced conformational change

Plasma proteins bind hormone without undergoing a conformational change through a static binding pocket formed between monomers or domains. For example, the tetrameric thyroid-binding plasma protein transthyretin forms a solvent-accessible hormone-binding channel at the oligomer interface^{35,36}. The structure of the protein is unchanged on binding hormone^{35,36}. By contrast, the structural role for ligand in the bound rTR α_1 LBD predicts that the receptor would differ in the bound and unbound states. In the absence of hormone, the receptor would possess a cavity at its core, uncharacteristic of a globular protein. Hormone binding is a dynamic process, which completes the hydrophobic core of the active receptor and induces an altered conformation^{1,2,9}.

An exact description of the hormone-induced conformational changes requires comparison of the structures of the liganded and the unliganded TR. The structure of the unliganded hRXR- α may substitute as a model for the unliganded TR¹⁰. The rTR α_1 LBD and hRXR- α LBDs adopt a similar fold, and it is possible that the structural similarity extends to the conformational changes.

There are three major differences between the two structures, which indeed appear related to hormone binding. First, the bound rTR α_1 LBD structure is more compact, with the hormone tightly packed within the core of the receptor. By contrast, the unliganded hRXR- α LBD contains several internal hydrophobic cavities, which is unusual in folded proteins. Two of these cavities were proposed as possible binding sites for 9-*cis* retinoic acid, although these sites only partly overlap with the single binding cavity observed in the liganded rTR α_1 LBD. The second difference involves H11 in the rTR α_1 LBD, which contributes to the hormone binding cavity. H11, continuous in the rTR α_1 LBD, is broken at Cys 432 in the RXR, forming a loop between H10 and H11 in the hRXR- α . This residue corresponds to His 381 in the TR, which provides a hydrogen bond to the outer ring hydroxyl of the ligand. Furthermore, the hormone binding cavity occupied by ligand in the rTR α_1 LBD is interrupted in the hRXR- α by the same loop, forming an isolated hydrophobic pocket in the RXR with H6 and H7. In the bound rTR α_1 LBD, the corresponding helices H7 and H8 are contiguous with the binding pocket, and enclose the hormone binding cavity from below.

The third difference between the two receptors is the position of the C-terminal activation domain. Although the C-terminal activation domain forms α -helices in both receptors, the domain in the rTR α_1 LBD follows a proline-rich turn, and lies against the receptor to contribute part of the binding cavity. In contrast, the activation domain in the unliganded hRXR- α is part of a longer helix which projects into the solvent.

These differences suggest a model for an alternative conformation of the TR LBD assumed in the absence of ligand. Perhaps in the unliganded TR, the subdomain of the receptor surrounding the hormone binding cavity is loosely packed, with the binding cavity occluded by a partly unstructured H11, providing a substitute core for the receptor. On binding hormone, residues which form a coil in the unbound receptor may engage the hormone, and continue H11. The ordering of H11 could unblock the hydrophobic cavity, allowing H7 and H8 to interact with hormone. The extended hydrophobic then collapses around the hormone, generating the compact bound structure.

It is difficult to predict the precise ligand-induced conformational changes in the C-terminal activation domain. Although

[§] Phasing power = $\langle F_o \rangle / \langle \epsilon \rangle$, where $\langle F_o \rangle$ is the mean calculated heavy-atom structure factor amplitude, and $\langle \epsilon \rangle$ is the mean estimated lack of closure.

^{||} $R_{\text{cullis}} = \langle \epsilon \rangle / \langle \text{iso} \rangle$, where $\langle \epsilon \rangle$ is the mean estimated lack of closure, and $\langle \text{iso} \rangle$ is the isomorphous difference.

^{*} $R_{\text{cryst}} = 100 \sum_{hkl} |F_o - F_c| / \sum_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factor amplitudes (for data $F_o > 2$). The R_{free} was calculated using 3% of the data, chosen randomly, and omitted from the refinement.

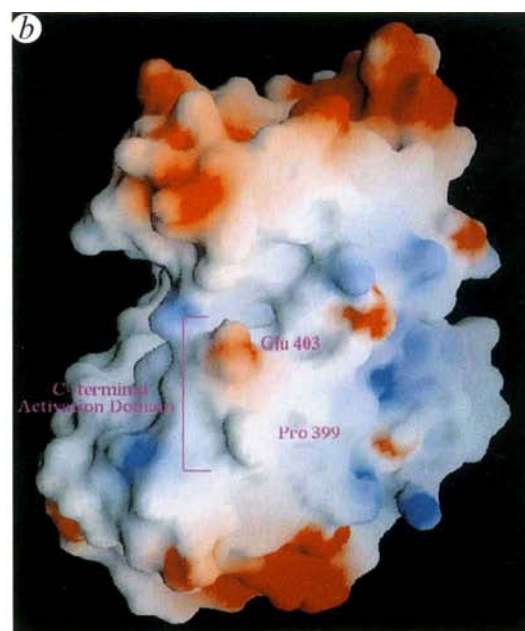
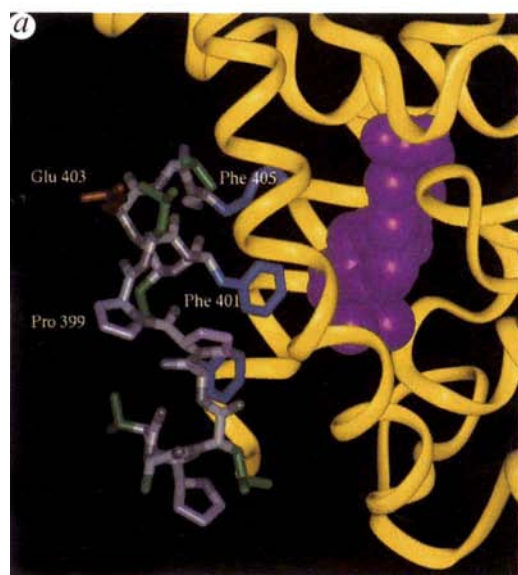


FIG. 5 *a*, Ribbon drawing of the $rTR\alpha_1$ LBD, showing the relation of the C-terminal activation domain to hormone. The view is identical to that of Fig. 3*a*. Residues that make up the C-terminal activation domain (Pro 393–Phe 405) are depicted as a stick representation. The domain forms an amphipathic helix, beginning at Pro 399 (grey). Hydrophobic residues, particularly Phe 401 and Phe 405 (blue), face inward towards the hormone. Glu 403 (red) projects outward into the solvent. Side

chains of other non-proline residues are green. *b*, Electrostatic potential surface of the C-terminal activation domain, calculated using GRASP⁴⁹. Negative electrostatic potential is red, positive electrostatic potential is blue. The C-terminal activation domain forms a largely hydrophobic surface, with the critical residue Glu 403 presented as a singular patch of negative charge.

the domain might assume an extended structure in the unliganded TR, and repack upon ligand binding, the change is probably more subtle, as the amino acid sequence of the $rTR\alpha_1$ in the turn (393–PTELFPP–399) significantly reduces its propensity to form an α -helix. It is remarkable that there is a continuous α -helix in the $hRXR-\alpha$ given the rather low helical propensity of the corresponding $hRXR-\alpha$ sequence (444–DTPIDT–449).

Nevertheless, it is likely that the conformation of the C-terminal activation domain in the bound structure differs from that in the unbound receptor. The packing density, defined as the number of atoms within 4.5 Å, is 1.5 σ below the mean for the activation domain as compared with the entire LBD, suggesting the domain packs loosely even in the bound structure. Moreover, most of the packing contacts for the C-terminal domain in the bound receptor are provided either by residues which interact with ligand, such as His 381, or by the ligand itself. Without the stabilization provided by hormone, it is likely that the C-terminal activation domain is disordered before hormone binding. The hormone-induced changes which affect H11 and H12 are reinforcing, and lead to the formation of the active conformation.

Mechanisms of hormone-dependent regulation

In mammalian cells, the TR commonly exhibits two activities: transcriptional repression in the unliganded state, and transcriptional activation in the liganded state⁹. A model for TR function proposes that these activities are mediated through hormone-dependent interactions with other proteins^{9,24}. The unliganded TR is thought to bind a co-repressor protein involved in repression of transcription^{37,38}. Ligand binding appears to induce both a dissociation of this protein with a relief of repression, and an association with another protein(s) that participates in the activation of transcription. Deletion of part of the C-terminal activation domain (Leu 402–Val 410) abolishes the ligand's influence on both activities: the truncated receptors bind ligand (albeit at a lower affinity), and undergo certain conformational changes in response to ligand binding, but are constitutive repressors²⁴. These observations imply that only a subset of the

conformational changes in the receptor that occur upon hormone binding influence of transcription by the receptor.

Ligand-induced changes in the conformation of the C-terminal domain, as discussed above, could influence its ability to interact with other proteins involved in mediating transcriptional regulation by the receptor. Inspection of the structure suggests that the partial deletion of the C-terminal activation domain would not compromise the structural integrity of the hormone-binding cavity, or prevent the formation of the compact bound state. An increased affinity of the C-terminal activation domain for other proteins could result from ordering of the domain, to present a more stable, and probably more extensive, interaction surface in the bound TR. Alternatively, ligand binding may release the domain from interactions within the LBD that obscure residues important for coactivator binding.

Ligand-induced changes can affect other receptor activities, such as dimerization. For example, hormone binding by the TR can modestly affect dimerization in a response-element-specific fashion^{39,40}. The $hRXR-\alpha$ forms a dimer through a surface provided by the N-terminal part of H10, together with H8 and H9¹⁰. Examination of $rTR\alpha_1$ LBD suggests that a similar surface might mediate TR dimerization. However, critical contacts with hormone are contributed by residues in the C terminus of H11. The direct participation of H11 in both processes is similar to the colocalization of ligand binding and dimerization in the oestrogen receptor, and suggestive of a central role for hormone in altering the dimerization surface upon ligand binding⁴¹. Such an alteration could be accomplished through a bend or rotation of H10, as may occur in the ordering and extension of H11 described above.

Conclusion

The structural role for ligand revealed by the bound $rTR\alpha_1$ LBD is fundamental to the mechanism of biological regulation of the receptor. Hormone controls receptor function through an absolute requirement for hormone in creating the active conformation of the receptor. Through a dynamic process,

hormone completes the hydrophobic core of the active conformation of the receptor, and directs the alignment of secondary structure elements critical for receptor function. We speculate that this process may resemble the registration of already formed secondary structure elements that occurs late in protein folding.

Given the sequence conservation within the LBD between members of the nuclear receptor superfamily, and the demon-

strated structural similarity, we suggest the binding mode observed here will be the prototype for the nuclear receptors. Although the mechanisms through which hormone exercises control over receptor function may differ among the nuclear receptors, the structural role for ligand, and its attendant conformational changes, will underlie these mechanisms. The implications of the structural role for ligand may explain the action of an entire class of hormones and receptors. □

Received 24 August; accepted 23 November 1995.

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ACKNOWLEDGEMENTS. We thank H. Nguyen for help in receptor expression; M. Bolger for providing the IpBr_2 ligand; J. Buchbinder, S. Gillmor and E. Sablin for help in synchrotron data collection; W. Weis for assistance with MADSYS; D. Freymann, P. Kushner, V. Ramalingam, T. Scanlan and A. Shiao for discussion; S. Gillmor, C. Honchell, T. Mau, R. Ribeiro and K. Yamamoto for critical reading of the manuscript. We also acknowledge the following beamlines, and their staff: Photon Factory BL6A2, NSLS X4A, CHESS F1 and especially SSRL 7-1. This work was supported in part by grants from the N.I.H. (J.B. and R.J.F.) and the Programs of Excellence in Molecular Biology (R.J.F.). R.L.W. is supported by an NSF predoctoral fellowship.

LETTERS TO NATURE

A water-vapour giga-maser in the active galaxy TXFS2226 – 184

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ACTIVE galactic nuclei are thought to be powered by gas falling into a massive black hole; the different types of active galaxy may arise because we view them through a thick torus of molecular gas at varying angles of inclination¹. One way to determine whether the black hole is surrounded by a torus, which would obscure the accretion disk around the black hole along certain lines of sight, is to search for water masers, as these exist only in regions with

plentiful molecular gas. Since the first detection² of an extragalactic water maser in 1979, they have come to be associated primarily with active galaxies, and have even been used to probe the mass of the central engine³. Here we report the detection of a water giga-maser in the radio galaxy TXFS2226 – 184. The strength of the emission supports a recently proposed theory of maser pumping⁴ that allows for even more powerful masers, which might be detectable at cosmological distances. Water masers may accordingly provide a way to determine distances to galaxies outside the usual distance ladder, providing an independent calibration of the Hubble constant^{3,5}.

Observations in the $6_{16}-5_{23}$ transition of water vapour (rest frequency 22.23508 GHz) were obtained during 4 April 1995, using the Max-Planck-Institut für Radioastronomie Effelsberg 100-m telescope in the position-switching mode. A K-band maser receiver was employed, the system noise temperature being ~100 K (including atmospheric contributions). The spectrometer was a 1,024-channel, three-level autocorrelator, providing 0.65 km s^{-1} sampling across a 50-MHz bandpass. The pointing accuracy was better than 10 arcsec (full-width, half-power beamsize, 40 arcsec). Absolute flux calibration of the H_2O emission was obtained through radio continuum measurements of NGC7027 and 3C286 (flux densities 5.9 and 2.45 Jy, respectively)^{6,7} after accounting for elevation-dependent gain variations and the frequency-dependence of the calibration signal in the receiver. Flux density uncertainties are of the order of $\pm 15\%$.

The spectrum (Fig. 1) shows H_2O maser emission with a peak flux density of $270 \pm 60 \text{ mJy}$ (see also Table 1). The maser, origi-

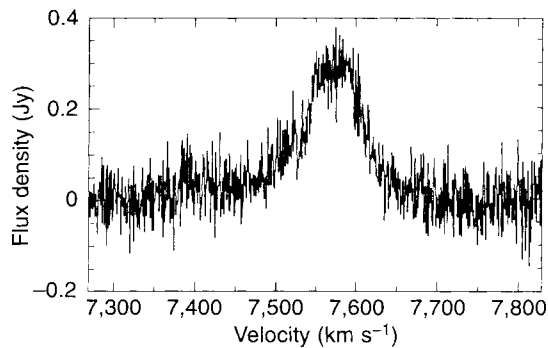


FIG. 1 H₂O spectrum towards TXFS2226–184 obtained on 4 April 1995, calibrated for good weather conditions to obtain reliable lower limits on the flux density. The spectrum has not been smoothed because the sampling interval (0.65 km s^{−1}) is close to the width of normal maser features (~1–5 km s^{−1}). The velocity is with respect to the local standard of rest, in the optical convention.

nally detected on 25 March 1995, has been further confirmed at three later epochs. Emission at 30-arcsec offsets in the four cardinal directions is consistent with that from a point source (size << 40 arcsec). The maser luminosity L , if emitted isotropically, is

$$\frac{L}{L_{\odot}} = 0.023 \left(\frac{\int S_{\text{H}_2\text{O}} dv}{\text{Jy km s}^{-1}} \right) \left(\frac{D}{\text{Mpc}} \right)^2 = 6,100 \pm 900$$

where $\int S_{\text{H}_2\text{O}} dv$ is the integrated profile flux density, $L_{\odot}$ is the solar luminosity and D is the distance ($D = 100$ Mpc, as derived from the optical emission line redshift $z = 0.025$, using $H_0 = 75$ km s^{−1} Mpc^{−1} and $q_0 = 0.1$). The error given is dominated by the uncertainty in the absolute flux calibration. The maser luminosity is about seven to eight orders of magnitude higher than that observed in a typical Galactic star-forming region. According to the current maser classification standard^{8,10}, the source should thus be designated the first known H₂O ‘‘giga-maser’’. We note that if the maser emission is anisotropic then this classification may not accurately describe its intrinsic luminosity. The recessional velocity is ~25% larger than that of any previously reported H₂O maser¹¹, and the isotropic luminosity is more than ten times higher than that of the next most luminous maser, NGC3079^{12,13}.

Optically, the maser is associated with an elliptical S0 galaxy. The emission-line spectrum displays line ratios [N II]/H α $\approx$ 0.8 and [O I]/[O III] $\approx$ 1, characteristic of a low-ionization nuclear emission region¹⁴ which is a signature of an active nucleus. In the far-infrared, the galaxy is associated with the IRAS source F22265–1826. Its 60- μ m flux (Table 2) is four orders of magnitude stronger than the far-infrared extension of the non-thermal radio power-law, indicating the presence of dust. The far-infrared luminosity $L_{\text{FIR}} \approx 10^{10} L_{\odot}$ is well below the level of a nuclear starburst¹⁵. Furthermore, the optical continuum spectrum is dominated by a ‘post-starburst’ stellar population (A.D.

and W.V.B., unpublished data). If triggered by a bar-like distortion of the gravitational potential, such a starburst leads to a massive inflow of gas toward the nuclear region¹⁶. The non-thermal 4.9 GHz radio emission (Table 2) is unresolved at ~1 arcsec ($\lesssim 0.5$ kpc) and hints at a compact nuclear object, whereas the radio power of the source is comparable to that of much more extended radio galaxies. Therefore, a likely model for the source involves the confinement of a powerful active galactic nucleus (AGN) by a dense surrounding medium of dust and gas.

Water masers have been previously observed in the nuclear regions of 16 active galaxies^{11,17,18} and are known to be confined to the central few parsecs in those three sources studied with high angular resolution^{3,8,19}. Recent observations of the active galaxy NGC4258 have shown that its maser emission arises in a warped, thin, edge-on gaseous disk or annulus at a galactocentric radius of ~0.2 pc (refs 3, 20, 21). Its keplerian rotation curve yields a central mass of a few times $10^7 M_{\odot}$, probably a massive black hole²². The masing gas may be heated to the required temperatures (300–1,000 K) by shocks from high-velocity flows^{23,24} or, more likely, by radiation from a compact nuclear X-ray source²⁵, with the warp allowing increased illumination of the disk. Using a collisional pumping model to simulate the inversion of the 6₁₆ and 5₂₃ H₂O states and finding that the masing zone is probably saturated, an intrinsic surface luminosity $\Sigma_{\text{H}_2\text{O}} \approx 10^{2.0 \pm 0.5} L_{\odot} \text{ pc}^{-2}$ is derived²⁵. This model can account for the observed maser luminosity of TXFS2226–184 if the illuminated surface area is 20–200 pc², yielding a maximum galactocentric radius of 2.5–7.5 pc for a disk-like structure.

Apparent H₂O luminosities well above the intrinsic luminosity can be caused by unsaturated maser emission efficiently amplifying the nuclear radio continuum along the line of sight. In this case the radiation is beamed into a narrow solid angle, equal to that subtended by the background source at the maser location. Assuming that the AGN core produces all the observed continuum and that the projected (unsaturated) masing region along the line of sight completely covers the continuum core, the required maser gain is ~20 for TXFS2226–184. In the case of incomplete coverage, the maser gain is larger.

In the X-ray illumination model (assuming the disk viscosity parameter, disk warping geometry and conversion efficiency of rest mass energy into X-rays to be the same as those inferred for NGC4258) the intrinsic luminosity scales as $L(\text{H}_2\text{O}) \propto L_{41}^{0.766} M_8^{1.234} L_{\odot}$, where $10^{41} L_{41} \text{ erg s}^{-1}$ is the 2–10 keV luminosity and $10^8 M_8 M_{\odot}$ is the mass of the central black hole¹. Although the central cores of radio galaxies with an obscuring torus will be intrinsically weak (no relativistic beaming), significant variations from source to source are also expected. Although the various parameters are poorly constrained, it has been predicted that the saturated emission model^{4,25} (and perhaps also the unsaturated maser scheme^{21,26}) imply the existence of maser sources far more luminous than those previously observed. TXFS2226–184 may be the first such object. The three lowest luminosity nuclear H₂O masers known are in the range ~ (0.1–1) $L_{\odot}$ (refs 27, 28). Our new detection therefore extends the luminosity range of H₂O masers in active galaxies to four orders of magnitude.

The different manifestations of energetic processes (radio jets, non-stellar optical and ultraviolet continuum, γ -ray emission) has provoked attempts to unify different classes of AGN. The presence of a pair of relativistic jets and a coaxial obscuring torus can yield drastically different views of the same object, depending on the viewing angle of the observer^{1,29,30}. In an attempt to unify the FRI radio galaxy–BL Lac and FRII radio galaxy–quasar–blazar schemes³¹, it has been suggested that the opening angle of the torus becomes wider when going from low-radio-power FRI to high-radio-power FRII class objects. For the same power of the central engine, spiral galaxies (containing Seyfert 1 and Seyfert 2 nuclei and most radio quiet quasars) appear to have more open circumnuclear tori than elliptical gal-

TABLE 1 Gaussian-fit parameters of maser profile

Right ascension (1950)	22 h 26 min 29.6 s
Declination (1950)	−18° 26′ 07″
S_{peak}^*	0.27 $\pm$ 0.06 Jy
$V_{\text{LSR}}^{\dagger}$	7,570 $\pm$ 1 km s ^{−1}
$\Delta v_{1/2}$ (FWHM)	88 $\pm$ 2 km s ^{−1}
$\int S_{\text{H}_2\text{O}} dv$	26.6 $\pm$ 0.6 Jy km s ^{−1}

* Peak flux density; the error represents uncertainties in the absolute flux calibration and not the smaller statistical error obtained from the gaussian fit.

[†] Velocity with respect to the local standard of rest, following the optical convention.

TABLE 2 Properties of TXFS2226–184

Optical redshift	z	0.025
Integrated magnitude	B	18.4
	V	17.4
	R	16.5
Flux density	12 μm	<0.12 Jy
	25 μm	<0.22 Jy
	60 μm	0.31 Jy
	100 μm	<0.57 Jy
	4.850 MHz	0.032 Jy
	1.490 MHz	0.070 Jy
	365 MHz	0.192 Jy
Spectral index α ($S_\nu \propto \nu^{-\alpha}$)	(365–4,850 MHz)	0.71 ± 0.02
Luminosity (60 μm)*		$2.5 \times 10^9 L_\odot$
Total FIR† luminosity*		$\leq 7.5 \times 10^9 L_\odot$

* Assuming $H_0 = 75 \text{ km s}^{-1} \text{ Mpc}^{-1}$, $q_0 = 0.1$.

† Far-infrared

axes (associated with FRI and FRII radio galaxies, BL Lac objects, and most radio-loud quasars). If this holds and if all luminous H_2O masers indeed arise from the innermost region, such differences may be highlighted by the measured H_2O line-shapes. An analysis of H_2O megamaser spectra and the profile shown in Fig. 1 is consistent with this expectation: most maser profiles are dominated by narrow spectral features (line widths $\sim (0.3\text{--}10) \text{ km s}^{-1}$). The only exceptions are the two early-type galaxies of the sample, NGC1052¹¹ and TXFS2226–184. Their maser profiles are composed of a single broad feature of width $\sim 100 \text{ km s}^{-1}$.

A major strength of this H_2O giga-maser detection lies in its potential. It may be not unreasonable to interpret the observed lineshapes towards NGC1052 and TXFS2226–184 in terms of saturated maser emission from a thick torus with a smaller ratio of binding mass to galactocentric radius than that found in NGC4258. However, very-long-baseline interferometry experiments are definitely required to elucidate the spatial fine structure of the masing gas (in TXFS2226–184, 1 pc corresponds to 0.002 arcsec). Both NGC1052 and TXFS2226–184 are marginally strong enough for such a detailed study. TXFS2226–184 is substantially more distant than NGC4258 and its recession velocity is much less affected by peculiar motions; therefore this galaxy may provide the best direct geometrical determination of Hubble's constant yet obtained. Detailed X-ray observations should also be obtained in order to constrain the mass of the central object.

The extreme luminosity of the H_2O maser in TXFS2226–184 raises the exciting prospect of detecting similar sources at much higher redshifts. At Effelsberg, sensitivity considerations yield a maximum attainable redshift for such a source of $z \sim 0.2$ (corresponding to a 5σ detection with a velocity sampling of $\sim 35 \text{ km s}^{-1}$ after a total integration time $\tau \approx 1 \text{ h}$). A deep search ($\tau \approx 25 \text{ h}$) could approach $z \approx 1$. Searches for H_2O masers in Seyfert galaxies are fairly complete to $z = 0.023$ and include several hundred objects (J. A. Braatz, A. S. Wilson and C. H., manuscript in preparation). Unpublished surveys of FRI and FRII radio galaxies up to $z \sim 0.15$ also exist but include a much smaller number of sources. In view of all these data it seems reasonable to suppose a space density of approximately one H_2O gigamaser within $z = 0.03$. If this holds, ~ 20 sources similar to TXFS2226–184 should be detectable up to $z \sim 0.15$, employing a 100-m radio telescope. Another interesting question relates to the upper end of the H_2O maser luminosity function. Ignoring possible differences between Seyfert (spiral) and radio or early-type (elliptical) galaxies and with $L(\text{H}_2\text{O}) \approx 500 L_\odot$ at $z = 0.004$ (NGC3079) and $L(\text{H}_2\text{O}) \approx 6,000 L_\odot$ at $z = 0.025$ (TXFS2226–184), we can speculate that there are masers of luminosity $\sim 10^5 L_\odot$ at $z \geq 0.1$. These would be sufficiently luminous to be observable at cosmological redshifts. Although such studies are of interest in estimating Hubble's constant and in

exploring the connection between galaxy type, redshift and enclosed nuclear mass, it is not currently possible to predict *a priori* whether a given Seyfert or radio galaxy will display maser emission. Nevertheless, the detection of an H_2O giga-maser in TXFS2226–184 is a significant step towards the investigation of stronger and more distant H_2O sources. $\square$

Received 11 May; accepted 15 November 1995.

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ACKNOWLEDGEMENTS. We thank the Max-Planck-Institut für Radioastronomie for their support in scheduling this project on the Effelsberg telescope. We also thank H. Falcke, C. Gwinn and C. Lonsdale for discussions. The work of W.V.B. was carried out at IGPP/LLNL with the support of the US Department of Energy.

Imaging the nonlinear grating in frequency-doubling fibres

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THE second-order response of a transparent material to intense light creates an oscillatory electromagnetic field at twice the driving frequency. Materials with a strong second-order response can therefore be used for frequency-doubling, for example to convert infrared laser light to visible light¹. Although amorphous materials have no significant intrinsic second-order response, glass fibres can nevertheless exhibit second-harmonic generation after exposure to intense laser irradiation². Beating between the electromagnetic fields of the laser light at the fundamental frequency and a weak second-harmonic signal (externally applied or intrinsic to the fibre) permanently modifies the glass and enhances the second-order response; the high efficiency of the response points to the formation of a periodic electric-field grating within the fibre^{3–7}. High electric fields have been detected in fibres⁸ and the existence of a grating has been confirmed indirectly⁹. Here we present direct images of this grating in germanosilicate optical fibres, obtained by exposing the fibres to chemical attack by hydrofluoric acid while the grating

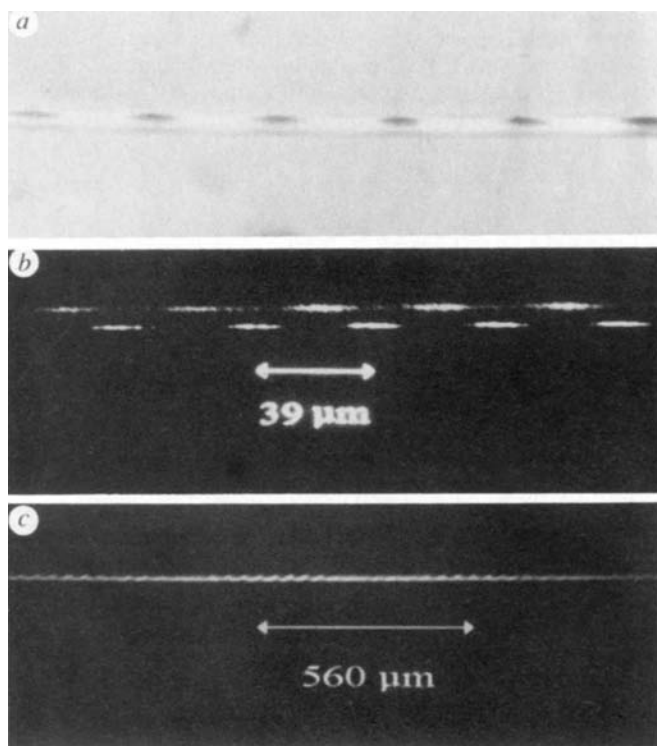


FIG. 1 Second-order nonlinear ($\chi^{(2)}$) gratings in frequency-doubling fibres revealed by chemical attack. Three P-codoped germanosilicate pieces of fibre were used, prepared by self-seeding (see text). *a*, Phase-contrast optical micrograph (magnification, $\times 240$). The period is $39\ \mu\text{m}$. The polarization of the grating is likely to be along the axis of the microscope. The region shown is 21 cm away from the input end. *b*, Etched frequency-doubling fibre showing alternating field polarity grating on top and bottom of the fibre. The brightness of the features on either side of the fibre was recorded with two independent settings for equalization. The polarization of the grating is likely to be perpendicular to the axis of the microscope; *c*, Superstructure of etched fibre, with period $\sim 0.56\ \text{mm}$.

is in place. The rate of etching is sensitive to the intensity of the internal electric field in the fibres. Our results are consistent with the idea that the grating results from macroscopic separation of charge at the boundary between the fibre core and cladding, rather than from a microscopic reorientation of dipoles throughout the material.

Efficient frequency doubling by a material requires not only a large intrinsic second-order response, but also matching of the phase velocities of the fundamental and second-harmonic electromagnetic waves to avoid the effects of destructive interference. The high efficiencies exhibited by laser-treated glass fibres indicate that the second-order susceptibility thus introduced varies periodically along the fibre, forming an electric-field grating that guarantees constructive interference^{3,7}; as the changes in the glass are determined by the relative phase of the beating fundamental and second-harmonic waves, the period of the grating thus established necessarily has the value required to compensate for any differences in phase velocity.

The observation of the grating on a micrometre scale has proved to be elusive up to now because the d.c. field is confined to the fibre core (typically $<5\ \mu\text{m}$ radius), and normally a much thicker cladding layer (typically $60\ \mu\text{m}$ radius) surrounds the core to protect it from the environment. The present technique is based on the fact that under an electric field distribution, etching by acids can become selective¹⁰. Recently, selective etching of borosilicate bulk glass exposed to light has been demonstrated¹¹. Here, it was expected that any periodic field distribution recorded in the core of the fibre could affect the

etching rate. The same acid was used for removal of the cladding of the fibres and for selectively etching the core, simplifying the experimental procedure. Initial tests were carried out with pieces of a B-codoped germanosilicate test fibre (fibre 1), immersed vertically in 1.5 cm steps into a 40% solution of HF for 10 min. The primary coating was removed before etching. Optical microscopy showed that a step-like profile resulted from etching, and the core was entirely removed after ~ 60 min exposure to HF. A nearly linear curve of fibre thickness versus etching time was obtained, and the rate measured was $1.11\ \mu\text{m}\ \text{min}^{-1}$. A similar procedure was used with another (P-codoped) germanosilicate optical fibre (fibre 2). The appropriate etching time for fibre 2 was found to be 52–54 min. All etching was carried out at room temperature (21°C).

The germanosilicate fibres used in the imaging experiments were single mode at $1.3\ \mu\text{m}$, and had the following characteristics. Fibre 1; 9 mol% Ge, co-doped with B, $125\ \mu\text{m}$ outer diameter, $9.4\ \mu\text{m}$ core diameter, and $1.38\ \mu\text{m}$ cutoff wavelength. Fibre 2; 3 mol% Ge, 0.5 wt% P doping, $120\ \mu\text{m}$ outer diameter, $8.8\ \mu\text{m}$ core diameter, and cutoff wavelength $1.30\ \mu\text{m}$. Standard telecommunication fibre with $8\ \mu\text{m}$ core diameter, $125\ \mu\text{m}$ outer diameter, and $1.25\ \mu\text{m}$ cutoff wavelength was also used. The pieces of fibre were prepared for frequency doubling using a Q-switched mode-locked Nd:YAG laser; the laser pulses were ~ 100 ps long, and the Q-switching rate was 1.2 kHz. Frequency doubling fibres can either be prepared by infrared radiation only at the fundamental frequency or with infrared and an external second-harmonic 'seed' wave^{2,3}. The first (self-seeded) method makes use of the second-harmonic radiation that builds up slowly along the fibre over many hours², which is believed³ to start from a weak second-harmonic signal generated by electric quadrupole interactions. This leads to gratings centred at ~ 20 cm from the input end. External seeding is much quicker and results in gratings close to the input end. Both preparation methods were used here. Two pieces of fibre 1 were prepared for four hours with average powers of $10\ \mu\text{W}$ second-harmonic and $70\ \text{mW}$ infrared; several pieces of fibre 2 were prepared with infrared injected light only (180 – $200\ \text{mW}$ average power) for ~ 10 hours, or alternatively for ~ 1 hour with the same infrared power and $1\ \text{mW}$ average power second-harmonic 'seed' radiation. The maximum second-harmonic signal measured was $1.1\ \text{mW}$ (for fibre 2). Although both fibres used were multimode at $0.53\ \mu\text{m}$, the fundamental transverse mode of propagation LP_{01} was the predominant mode of the second-harmonic signal.

All pieces of prepared fibre that were etched showed a periodic structure, to a varying degree of modulation. Unprepared fibres were etched under the same conditions and maintained a cylindrical form. Figure 1*a* shows an example of a phase-contrast microscope picture of a prepared piece of fibre 2. The selective etching is clearly seen, and the grating period is determined to be $39\ \mu\text{m}$, while the thickness of the fibre after etching is $7\ \mu\text{m}$. The period measured for fibre 1 was $37\ \mu\text{m}$. Even pieces of the standard (germanosilicate) telecommunications fibre prepared for frequency doubling showed the grating after etching. The gratings were found to be as long as several (~ 10) centimetres. The grating location and distribution, as measured by direct inspection under a microscope, agreed with results obtained previously by macroscopic characterization techniques such as cut-back and heat scan^{12,13}. It was expected that the regions of enhanced etching should alternate between the top and bottom of the fibre, as the field polarity alternates³. This feature is illustrated in Fig. 1*b*, where the brightness of the top and bottom of the photo was adjusted independently. An electron scanning microscope was used to study an etched fibre, and the periodic modulation of the diameter of the fibre was found to be $<0.1\ \mu\text{m}$, in spite of the fact that the optical microscope clearly showed the grating. Some of the fibres examined also showed a superstructure of period $\sim 0.56\ \text{mm}$, as illustrated in Fig. 1*c*, consisting of an amplitude modulation of the pattern that appeared after

etching. Because of the relatively high spatial frequency, its origin is probably related to beating between modes.

The physical mechanism of selective etching was studied by etching bulk silicate glass samples with high internal electric fields created by thermal poling according to the procedure described in ref. 14. The 1-mm-thick samples were heated at 300 °C for 60 min with 3 kV applied between the central part of the top and bottom faces. As in ref. 14, such procedure led to the recording of a permanent second-order nonlinearity in the poled region of the glass, where a high electric field region was recorded. The samples were then immersed in 40% HF for 4 min and the etching rate was found to be different for the poled and unpoled parts of the glass. Between the regions of high electric field (poled glass) and zero electric field (unpoled), a step with a sharp edge ($\sim 0.6 \mu\text{m}$ deep) was created by an unequal etching rate. This implies that a nonuniform charge distribution modifies the etching rate, and is sufficient to account for the images of the gratings shown in Fig. 1 even in the absence of the optical preparation process used in fibres. Increasing the etching time in poled bulk glasses five times increased the depth of the step to only $\sim 1 \mu\text{m}$. Once the field source was removed by etching, the etching rate became equal to that of unpoled glass. Furthermore, the edge became blurred, indicating that the contrast of the pattern engraved in the glass becomes poorer once the source of the strong field is removed by etching.

In fibres, removal by etching of the outer layer of the core can give an insight into the physical processes involved in grating formation. As discussed for bulk samples in ref. 15, if the grating were written by orientation of dipoles, the source of the electric field is distributed across the core. Therefore, etching deep in the core should result in deep modulation. In contrast, if the field were caused by macroscopic charge separation to the core-cladding boundary, removal of the outer layer of the core should remove all dislocated charge. Etching deep into the core should blur the image. Deliberately overexposing the grating to HF showed that the contrast of the grating viewed by microscopy became poorer once the core-cladding interface was removed, consistent with the macroscopic charge-separation mechanism. Insight can also be gained by determining the maximum fibre thickness (minimum etching) for grating visualization. Various pieces of frequency-doubling fibre of gradually varying thickness were obtained by slowly immersing the fibres into HF. Gratings could be imaged in fibre 2 ($4 \mu\text{m}$ core radius) for a diameter as large as $14 \mu\text{m}$, implying that selective etching starts to take place at least $3 \mu\text{m}$ before the acid reaches the core-cladding interface. This long-range effect is likely to be a measure of the charge or electric-field distribution in prepared fibres. The present technique is adequate for microscope studies such as the phase of gratings, beating between modes, and erasure by light or by temperature of fibres prepared under various conditions of exposure to infrared, visible and ultraviolet radiation. $\square$

Received 5 June; accepted 8 November 1995.

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ACKNOWLEDGEMENTS. We thank R. R. Aveliz and M. F. S. Lopes for use of microscope facilities, and E. N. Hering, F. C. Garcia and M. L. Fernandez for experimental help with etching and poling experiments. This work was supported by the Conselho Nacional de Pesquisa (Brazil) and NUTEK (Sweden).

A room-temperature organometallic magnet based on Prussian blue

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THE rational design of molecular compounds that exhibit spontaneous magnetic ordering might enable one to tailor magnetic properties for specific applications in magnetic memory devices^{1–4}. In such materials synthesized previously^{5–17}, however, the underlying weak magnetic interactions are incapable of maintaining ordering at ambient temperatures. One remarkable exception is a compound derived from vanadium and tetracyanoethylene¹⁸, but the material is amorphous and fragile, and consequently the molecular interactions responsible for its striking properties are not understood. Here we demonstrate another route to the synthesis of a room-temperature organometallic magnet, in which we combine a hexacyanometalate $[\text{M}(\text{CN})_6]^{n-}$ with a Lewis acid L^{p+} . If L and M are transition-metal ions, then the orbital interactions in the resulting compound can be described by well understood principles^{21–24}, and it is therefore possible to choose the metals to tune the compound's magnetic properties—in particular, the magnetic ordering (Curie) temperature T_c (refs 21–26). We have synthesized a room-temperature magnetic material ($T_c = 315 \text{ K}$) that belongs to the Prussian blue family of compounds²⁷ (where M is chromium and L is vanadium), demonstrating that transition-metal hexacyano complexes are promising components for the construction of molecule-based high- T_c magnets.

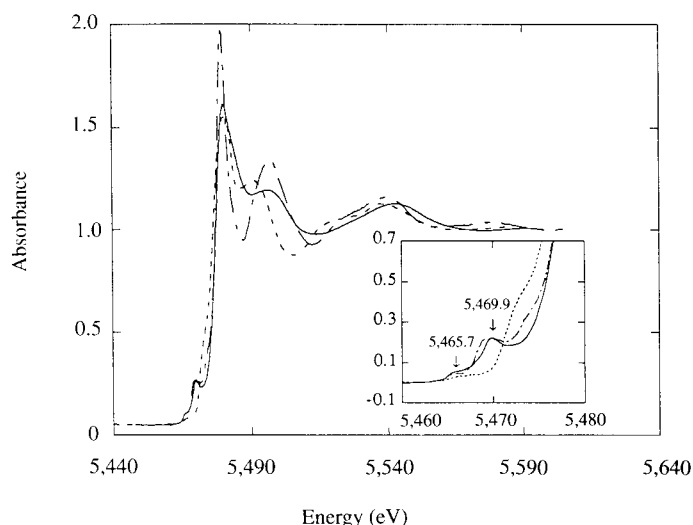


FIG. 1 X-ray absorption near-edge structures at the vanadium K edge of compound **1** (---), $\text{K}_4[\text{V}^{\text{II}}(\text{CN})_6]$ (— · —) and $(\text{NH}_4)_2[\text{V}^{\text{II}}(\text{H}_2\text{O})_6](\text{SO}_4)_2$ (.....). X-ray absorption spectroscopy³¹ is element-sensitive and gives independent information on the local order around each of the absorbers: electronic structure from the near-edge structures (0–150 eV after the pre-edge) and the radial distribution function of the neighbours around the absorber from the extended X-ray absorption fine structures (0–1 keV). Efficient for all states of sample (solid, surface, liquid, gas), it is well adapted to amorphous compounds. All three spectra present a first transition, corresponding to a $1s \rightarrow 3d$ excitation, with a weak intensity—in line with a quasi-octahedral surrounding of the vanadium—and at the same energy ($\sim 5,465.7 \text{ eV}$), characteristic of vanadium(II).

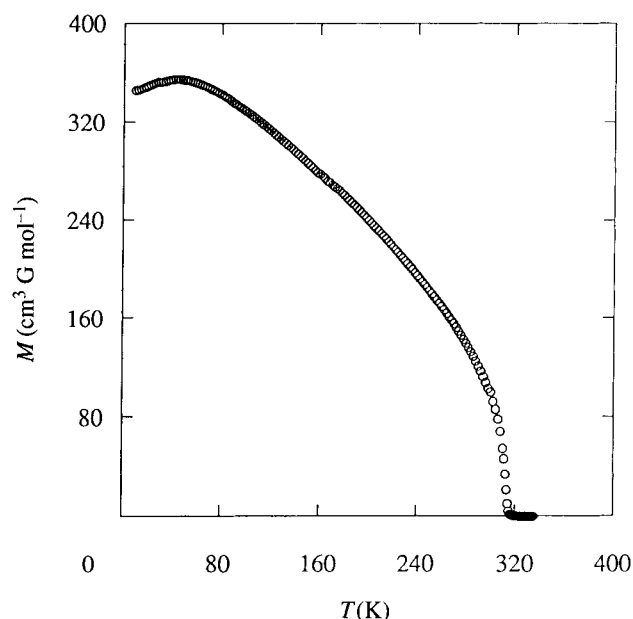


FIG. 2 Thermal dependence of the magnetization of compound **1** in a low applied field ($H = 10$ G). The sharp increase of the magnetization at $T_c = 315$ K is noteworthy.

The dark blue solid, $V[Cr(CN)_6]_{0.86} \cdot 2.8 H_2O$, hereafter designated compound **1**, is obtained by mixing quickly aqueous solutions of potassium hexacyanochromate(III), $K_3[Cr^{III}(CN)_6]$, and Tutton salt $(NH_4)_2V^{II}(H_2O)_6(SO_4)_2$ under anaerobic conditions in a gas ramp. A precipitate occurs from the deep blue solution. The solid is filtered quickly, washed with water, dried under vacuum and stored in sealed oxygen-free vessels. Compound **1** is air-sensitive but when dispersed in paraffin oil or mixed with glue it shows stability in air and maintains its magnetic properties for weeks.

Compound **1** is amorphous; its X-ray powder diffraction diagram is featureless. The proposed formula fits well the elemental chemical analysis. The number of water molecules is confirmed by thermogravimetric analysis between 20 and 220 °C. The formula is different from that expected if all vanadium remained bivalent ($V_3[Cr(CN)_6]_2$) (ref. 20). Because the vanadyl $V^{IV}=O$ band is not detected in the infrared spectrum (see below), the electric balance requires vanadium(II) to be partly oxidized into vanadium(III) (that is, $V_{0.42}^{II}V_{0.58}^{III}[Cr^{III}(CN)_6]_{0.86} \cdot 2.8 H_2O$). The compound is non-stoichiometric: different preparations using the same synthetic conditions lead to slightly different ratios $\rho[Cr(CN)_6/V]$ ($0.82 < \rho < 0.94$). The Curie temperatures are always close to 315 K (± 5 K). Different synthetic conditions lead to large changes in composition and in magnetic properties. Slow addition of the reactants, generally used in order to obtain better-crystallized materials in Prussian blue derivatives, facilitates the oxidation (probably by the solvent) of V^{II} to V^{III} and $V^{IV}O$ and to a decrease in T_c . The final well-defined vanadyl derivative has $T_c = 115$ K. Some preparations contain sulphate and ammonium as traces. The infrared spectrum shows the presence of coordinated water (at $\sim 3,500$ cm^{-1}). The 2,000–2,200 cm^{-1} region is sensitive to the $C\equiv N^-$ stretching vibration and therefore to the bonding mode of the cyanide^{19,28}. For compound **1** it shows a composite asymmetric band centred on 2,104 cm^{-1} . This value is lower than with bivalent ions with larger atomic numbers Z , probably owing to higher energy and larger spread of the V^{II} orbitals and to the correlated enhanced $d-\pi^*$ antibonding interaction. Because under air oxidation the band becomes symmetric at 2,118 cm^{-1} ($Cr^{III}-C\equiv N-V^{III}$) and then shifts to 2,170 cm^{-1} ($Cr^{III}-CN-V^{IV}O$), it can be assigned to a $-Cr^{III}-C\equiv N-(V^{II}-V^{III})$ sequence.

Local structural information is also provided by X-ray absorption spectroscopy (see Fig. 1). The technique was used at the K edge of chromium and vanadium. EXAFS at the chromium K edge shows that compound **1** and $K_3[Cr(CN)_6]$ have the same first shell (six carbon neighbours, Cr–C distance = 2.05 ± 0.02 Å) and the same second shell (six nitrogens), whereas a third shell of heavy neighbours (V) appears only in **1**, at about 5.3 Å; at the vanadium K edge, three shells of neighbours are present. The first shell corresponds to a mixture of V–nitrogen (from cyanide) and V–oxygen from coordinated water (V–nearest neighbours mean distance = 2.13 ± 0.03 Å). A heavy neighbour (Cr) is visible at ~ 5.3 Å. The pre-edges at the chromium K edge of compound **1** and of $K_3[Cr(CN)_6]$ are superimposable. The vanadium K edge spectra of compound **1** and two vanadium(II) models (Fig. 1) demonstrate the presence of vanadium(II) (ref. 29). The data are fully consistent with the presence in **1** of the molecular precursor $[Cr(CN)_6]^{3-}$, of the $Cr-C\equiv N-V$ sequence (distance Cr–V ≈ 5.3 Å), with $[Cr(CN)_6]$ vacancies filled with water molecules coordinated to vanadium as in Prussian blue systems with a M/L stoichiometry less than 1 (ref. 20). It is therefore reasonable to propose from the spectroscopic and structural data that short-range order in compound **1** is similar to the rock-salt type structure of Prussian blue²⁰. The small size of the particles, due to quick mixing of the reactants, precludes an extended long-range structural order.

The thermal dependence of the magnetization is shown in Fig. 2. The transition temperature is observed at $T_c = 315$ K: compound **1** is a room-temperature magnet. Figure 3 shows a plot of magnetization against applied field at $T = 10$ K: Fig. 3a shows the saturation magnetization at 0.15 Bohr magneton; Fig. 3b displays the hysteresis loop at $T = 10$ K.

The magnetic properties can be understood from the spectroscopic and structural data. The low value of the saturation magnetization M_s at 10 K implies that the Cr^{III} electrons on the one hand, and the V^{II} and V^{III} electrons on the other, have their spins antiparallel, which implies a short-range antiferromagnetic interaction between them. With this spin arrangement, the computed value ($M_s = 0.16$ Bohr magneton; see Fig. 3a) is in fair agreement with the measured value (0.15). It corresponds to an exact cancellation of the spins of Cr^{III} and V^{II} ($|S_{Cr^{III}}| = |S_{V^{II}}| = 3/2$) and to non-compensated spins of Cr^{III} and V^{III} ($S_{Cr^{III}} = 3/2$ and $S_{V^{III}} = 1$). Parallel alignment of the spins (known as ferromagnetism) would give rise to a much higher magnetization value.

Long range order of antiferromagnetically coupled, non-compensated spins is known as ferrimagnetism. Why compound **1** orders as a ferrimagnet at a T_c higher than room temperature, despite spin bearers at a distance ~ 5.3 Å, can be understood from the geometry and the electronic structures of chromium and vanadium.

According to Néel³⁰, T_c in ferrimagnets is proportional to the number of magnetic neighbours around a given metallic centre and to the magnitude of the interaction between two centres. In a perfect Prussian blue structure with a $V/Cr_{0.86}$ stoichiometry, it can be shown that there are 5.16 chromiums around each vanadium; symmetry accounts for the antiferromagnetic nature of the interaction between unpaired electrons of the metal ions²²: in O_h symmetry, the unpaired electrons of chromium (three) and vanadium (three for V^{II} and two for V^{III}) are described by d wave functions of the same π symmetry (called t_{2g} orbitals in group theory). The orbitals overlap in the linear $-Cr-C\equiv N-V-$ μ -cyano sequence; and lead to antiferromagnetic interactions (as usual in chemical bonding, the larger the overlap the larger the interaction). Indeed, we chose vanadium(II) to react with $[Cr^{III}(CN)_6]^{3-}$ to get a particularly strong antiferromagnetism: (1) there is a good match in energy and a large overlap between the t_{2g} electrons of chromium and vanadium (vanadium orbitals are more diffuse than those of cations with higher atomic numbers); (2) there are no unpaired electrons in the vanadium σ -symmetry orbitals (called e_g orbitals) and there is

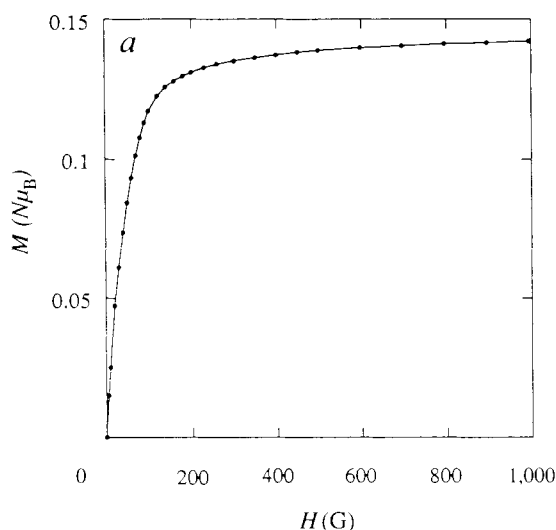
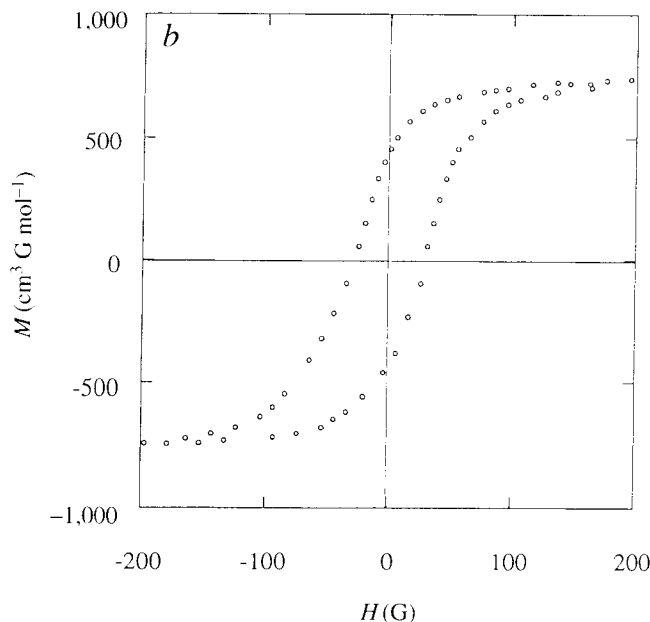


FIG. 3 a, Plot of magnetization against applied field up to $H=1$ kG of compound **1** at $T=10$ K. Magnetization increases rapidly up to 200 G and then tends slowly to its saturation value $M_s \approx 0.15$ Bohr magneton, assuming a mean Lande g factor value = 2. The experimental saturation magnetization compares well with the computed value, using the V^{II} and V^{III} formula weight and antiferromagnetic coupling between chromium and vanadium: $M_s = |Ng\mu_B S_{\text{total}}|$ (N , Avogadro constant; μ_B , Bohr

therefore no ferromagnetic interaction between electrons in orthogonal orbitals (as in the dioxygen molecule, or as in $\text{Cs}^{\text{I}}\text{Ni}^{\text{II}}[\text{Cr}^{\text{III}}(\text{CN})_6]$ (ref. 21). The number of magnetic neighbours and the strong antiferromagnetic interaction between non-compensated spins explain the high T_c . The three-dimensional ferrimagnetic approach illustrated by our result seems to be one of the most promising routes for designing further high- T_c magnets.

Although the interest of this finding is currently scientific rather than practical, one of the challenges with molecule-based magnets is to find applications as a result of their specific properties such as low density, transparency, insulating character, magneto-optical properties and tunable Curie temperatures¹⁻⁴. We have already used the room-temperature Curie point, the sharp increase of the magnetization at T_c and absence of observable fatigue or deterioration during thermal cycles around the transition to design a temperature-sensitive magnetic switch. □



magneton); $S_{\text{total}} = [0.86 \times S_{\text{Cr}^{\text{III}}} - [0.42 \times S_{\text{V}^{\text{II}}} + 0.58 \times S_{\text{V}^{\text{III}}}]$. With $S_{\text{Cr}^{\text{III}}} = 3/2$, $S_{\text{V}^{\text{II}}} = 3/2$, $S_{\text{V}^{\text{III}}} = 1$ and $g = 2$, $M_s = 0.16$ Bohr magneton. b, Hysteresis loop in the ± 200 G range at $T=10$ K. The remanent magnetization is $M_r = 457 \text{ cm}^3 \text{ G mol}^{-1}$ and the coercive field is $H_c = 25$ G.

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ACKNOWLEDGEMENTS. Our X-ray absorption data were recorded at LURE, the French synchrotron facility at Orsay, in collaboration with F. Villain and C. Héary.

Selective binding and removal of guests in a microporous metal–organic framework

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MICROPOROUS inorganic materials such as zeolites find widespread application in heterogeneous catalysis, adsorption and ion-exchange processes. The rigidity and stability of such frameworks allow for shape- and size-selective inclusion of organic molecules and ions¹⁻⁴. Analogous microporous structures based on organic building blocks have the potential for more precise rational design, through control of the shape, size and functionalization of the pores⁵⁻⁸. Here we report the synthesis of a metal–organic framework designed to bind aromatic guest molecules selectively. The basic building block is a symmetric organic molecule, which binds metal ions^{9,10} to form layers of the metal–organic compound alternating with layers whose composition is determined by the functionalization of the starting molecules. The layers create channels in which guest aromatic molecules may be selectively bound. We show that the crystal lattice thus formed is thermally stable up to 350 °C, even after removal of included guest molecules, and that the inclusions can be selectively readsorbed.

Received 21 April; accepted 25 October 1995.

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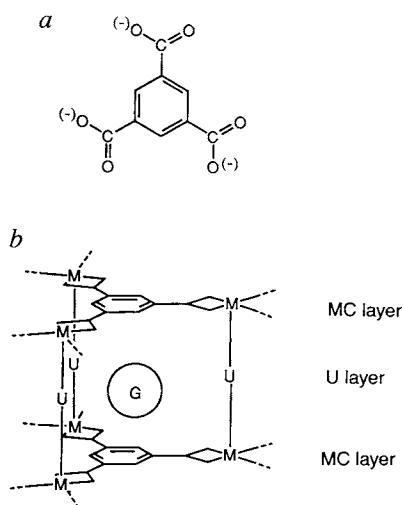


FIG. 1 a, Structural formula 1,3,5-benzenetricarboxylate (BTC), and b, its coordination to a metal ion to form alternating metal-carboxylate and spacer unit layers to form voids where guest inclusions are accommodated.

Our construction strategy uses symmetry and functionality of the starting molecular building block in its reaction with metal ions to support the formation of a covalent solid having the desired open framework^{9,10}. Multidentate molecular components are used to enhance its thermal stability. We chose 1,3,5-benzenetricarboxylate (BTC) (Fig. 1a) as the building block as it has three equally spaced carboxylate groups linked to an aromatic ring to give it a rigid disk-like conformation. As carboxylate groups bind to transition metal ions within the same plane as either bidentate or monodentate ligands¹¹, the reaction of BTC with a first-row transition metal should yield a material composed of metal-carboxylate (MC) layers (Fig. 1b). We considered that these sheets might be made functional by using a method previously applied to layered vanadium phosphates¹² and lead halides¹³, in which other molecular units (U) are introduced to occupy vacant metal sites lying perpendicular to the layers. The stacking of these layers in the crystal should produce alternating MC and U layers and, depending on the choice of U, a suitable environment for selective inclusion of guests (G) between the MC layers.

The success of this synthetic strategy was demonstrated by diffusing pyridine into mixture of the acid form of BTC and $\text{Co}(\text{NO}_3)_2$ in alcohol for three days to give a good yield of large, pink, cubic crystals which were insoluble in water and common organic solvents. The homogeneity of the bulk product was confirmed by comparison of the observed and calculated powder diffraction patterns obtained from single-crystal data. An X-ray crystal analysis on a single crystal from this reaction revealed the presence of a neutral porous network represented as $\text{Co}_6\text{H}_3(\text{COOH}_{1/3})_3(\text{NC}_5\text{H}_5)_2 \cdot 2/3 \text{NC}_5\text{H}_5$. A fragment of the structure is shown in Fig. 2, where three BTC units are coordinated to each of the Co(II) centres. One of the BTC units is completely deprotonated and coordinates to three metal centres in a bidentate fashion, whereas each of the other units coordinates to three metal centres as monodentate ligand; the remaining free carbonyls hydrogen-bond strongly to adjacent free carbonyls. The remaining positions on the metal centres are occupied by pyridine molecules, which we treated as being statistically disordered, with two preferred orientations around the Co-N bond. This arrangement generates extended sheets along the x - y plane. In the crystal these sheets stack along the z -axis to give alternating cobalt-carboxylate and pyridine layers (Fig. 3a). The cobalt-carboxylate layers are separated by 7 Å. The anchored pyridine ligands hold these layers tightly together by

mutual π -stacking, as is evident from the distance (4–6 Å) between pyridines. In the remaining space between the sheets there are uncoordinated pyridine molecules which occupy the rectangular channels (7×10 Å) that result from the tight fit between the layers. This gives the structure a three-dimensional character: in the direction perpendicular to the layers, the closely interacting pyridines form a rigid channel system. The separation between the layers and the channel structure remain unaltered when guests are removed or included (see below). It is therefore reasonable to consider this compound as being analogous to zeolites rather than to intercalation compounds. The pyridine guests π -stack with the benzene rings of BTC units of adjacent layers (Fig. 3b), where the distance between the pyridine π -system and that of BTC is 3.5 Å.

The π -stacking of both the coordinated pyridine ligands and the included pyridine guests have significant implications for the rigidity and selectivity of this porous framework. Thermal gravimetric analysis of a crystalline sample showed a weight loss of 11.7% at 190 °C, corresponding to the loss of the pyridine guests occupying the channels ($2/3 \text{NC}_5\text{H}_5$ per formula unit), followed at 350 °C by a further weight loss (45.5% of total), corresponding to the loss of the remaining pyridine molecules

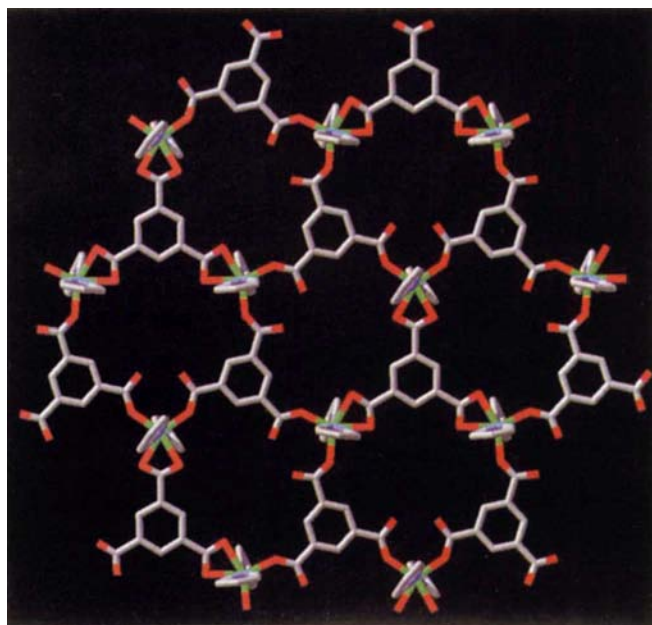
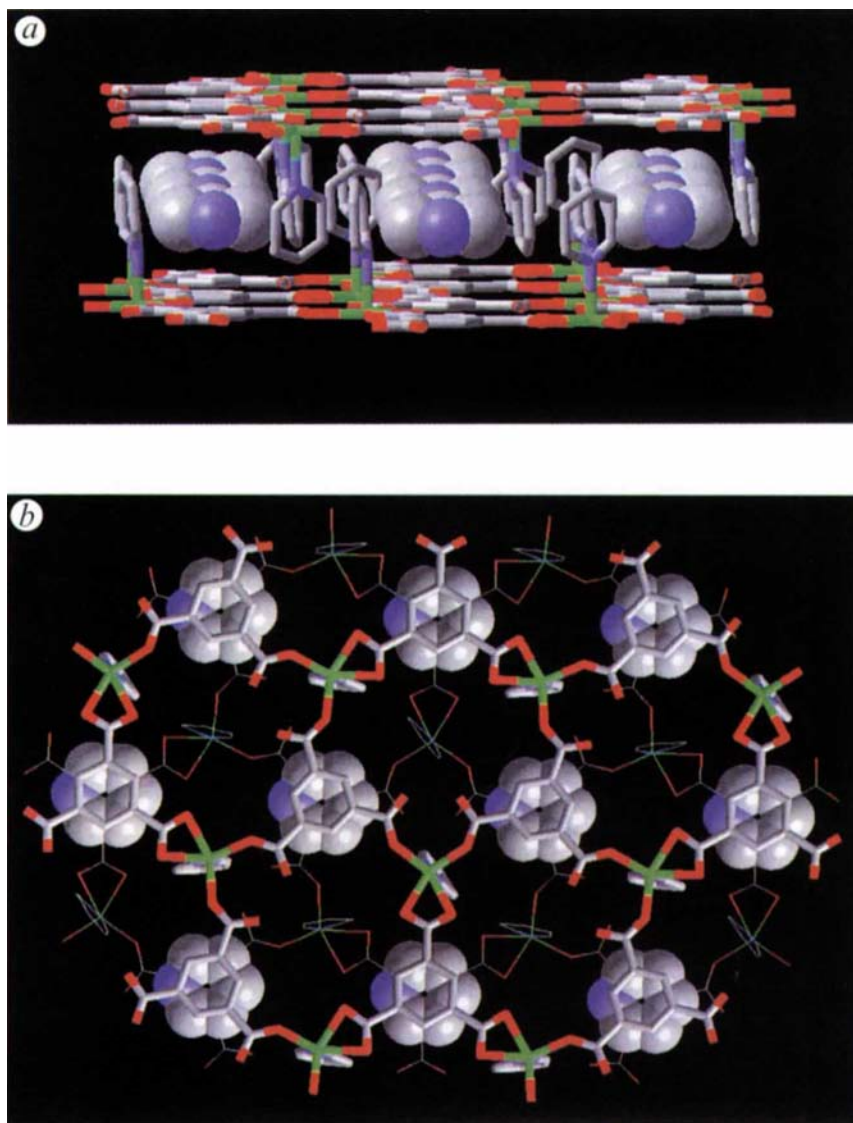


FIG. 2 A single layer of the extended porous network of $\text{Co}_6\text{H}_3(\text{COOH}_{1/3})_3(\text{NC}_5\text{H}_5)_2 \cdot 2/3 \text{NC}_5\text{H}_5$. The metal-carboxylate layer is shown approximately along the x - y plane: green, Co; red, O; blue, N; grey, C. Only one of the two preferred orientations about the Co-N bond is shown for the statistically disordered coordinated pyridine molecules. The hydrogen atoms on the pyridines and BTC units are omitted for clarity.

METHODS. Single crystals of this material at 20 ± 1 °C are hexagonal, of space group $P6_3/mcm-D_{6h}^{3-}$ (no. 193), with $a = 16.711(4)$ Å, $c = 14.189(2)$ Å; $V = 3,423(1)$ Å³ and $Z = 6$ for $x = 1$, $d_{\text{calc}} = 1.464$ g cm⁻³, μ_a (MoK α) = 0.80 mm⁻¹. A total of 1,161 independent reflections having 2θ (MoK α) < 50.7° (the density of crystals freshly isolated from the mother liquor was measured by flotation as 1.48 ± 0.01 g cm⁻³). Elemental microanalysis on crystalline samples of this material gave C, 54.99; H, 3.82; N, 7.44; Co, 12.43%; calculated values for $\text{Co}_6\text{H}_3(\text{COOH}_{1/3})_3(\text{NC}_5\text{H}_5)_2 \cdot 2/3 \text{NC}_5\text{H}_5$ were C, 56.11; H, 3.66; N, 7.82; Co, 12.33%.

FIG. 3 *a*, A perspective drawing of the solid-state structure of $\text{CoC}_6\text{H}_3(\text{COOH}_{1/3})_3(\text{NC}_5\text{H}_5)_2 \cdot 2/3 \text{NC}_5\text{H}_5$ perpendicular to the *z*-axis. The cobalt-carboxylate layers with anchored pyridines are shown as line drawings for clarity. Pyridine guest molecules are represented by their space-filling van der Waals radii to show their occupation of the channels within the structure. *b*, View of the structure along the *z*-axis showing π -stacking of the pyridine guests with the benzene rings of BTC units. For clarity, the top layer is depicted in thicker lines than the lower layer. The colour scheme is the same as in Fig. 2. The pyridine guests in *a* and *b* were refined as six-membered carbon rings and centred about the $-3m$ site at the origin of the unit cell and the 32 site at $(2/3, 1/3, 0)$ (further details available from the authors). Their representation as pyridine molecules and their orientation in the face-to-face mode with BTC is shown for clarity.



bound to the metal centres within the framework (2 NC_5H_5 per formula unit). This material remains crystalline upon removal of the pyridine inclusions. The X-ray powder pattern of a sample that has been heated to 200°C for six hours shows that the positions and intensities of the most intense lines (002), (003), (004), (005) and (006) remain unchanged relative to those for an unheated sample of the solid. Elemental microanalysis for the heated material (measured values for C, 52.38; H, 3.46; N, 6.84%; calculated values for $\text{CoC}_6\text{H}_3(\text{COOH}_{1/3})_3(\text{NC}_5\text{H}_5)_2$ were C, 53.66; H, 3.32; N, 6.59) points to the absence of pyridine guests. Further heating of this sample to 350°C reveals the absence of pyridine in the sample and a different X-ray diffraction pattern for the resulting solid. However, upon addition of pyridine the original pattern for the solid as synthesized is obtained. Presumably, the cobalt-carboxylate layer is preserved throughout the cycle of heating, cooling and inclusion. We are investigating this further to confirm the stability of the cobalt-carboxylate layer.

Examination of the single crystals with an optical microscope at 50°C under a pressure of 0.001 mm Hg showed that they retain their morphology and crystallinity upon loss of the pyridine guests. We used infrared spectroscopy to show that solid samples of this material, from which the pyridine guests had been removed, selectively absorb aromatic molecules such as benzene, nitrobenzene, cyanobenzene and chlorobenzene, but not acetonitrile, nitromethane or dichloroethane. These experi-

ments were done by suspending the solid in a solvent with aromatic and non-aromatic components; for example, 0.5 g solid was added to 1 ml of a 1:1 mixture of $\text{C}_6\text{H}_5\text{CN}/\text{CH}_3\text{CN}$ as solvent and allowed to stand for less than 30 min. After filtration and drying, a solid containing $\text{C}_6\text{H}_5\text{CN}$ but no CH_3CN was obtained. The Fourier transform of the infrared spectrum of the resulting solid showed a peak at ν_{CN} at $2,231\text{ cm}^{-1}$, corresponding to that of $\text{C}_6\text{H}_5\text{CN}$, and nothing in the range $2,240$ – $2,260\text{ cm}^{-1}$, where aliphatic non-conjugated nitriles like acetonitrile are expected to appear. Analogous experiments for 1:1 mixtures of the solvents $\text{C}_6\text{H}_5\text{NO}_2/\text{CH}_3\text{NO}_2$, $\text{C}_6\text{H}_6/\text{CH}_3\text{NO}_2$ and $\text{C}_6\text{H}_5\text{Cl}/\text{C}_2\text{H}_5\text{Cl}_2$ showed that the solid consistently had greater selectivity for inclusion of the aromatic solvent. In each case the diffraction pattern of the resulting sample was similar to that of the original material—the line positions remain unchanged, although the intensities changed slightly. This indicates that the crystal lattice is preserved and the distance between the cobalt-carboxylate layers is constant. We believe that the remarkable selectivity of this open framework towards aromatic molecules is a direct consequence of their π -stacking with the BTC units within the sheets. The use of multidentate ligands such as BTC coupled with the functionalization of the framework contributes greatly to the unusual thermal stability of this material. In conclusion, our results demonstrate the application of molecular chemistry in the design of thermally stable open and rigid frameworks capable of selective inclusion. □

Received 22 June; accepted 31 October 1995.

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ACKNOWLEDGEMENTS. The financial support of the NSF, the Exxon Education Foundation and the Materials Research Group at Arizona State University are acknowledged. We thank E. Houseman for her assistance in producing Fig. 1.

Influence of mountain ranges on the mid-latitude atmospheric response to El Niño events

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TROPICAL heating associated with El Niño events influences weather patterns around the globe^{1–4}, in part by generating wave-like disturbances of vorticity (a measure of local fluid circulation about the vertical) in the upper troposphere which extend into the mid-latitude regions. But these waves do not account well for the observed mid-latitude consequences of El Niño^{5–8} events. Here we show that a secondary interaction of these waves with mid-latitude mountains contributes significantly to the observed flow patterns. On encountering a mountain, a column of rotating air is compressed vertically, spreads horizontally, and its net vorticity is thereby reduced. For a realistic distribution of El Niño-related tropical heating, we find that vortex compression, primarily in the Himalayan–Tibetan region, generates a vorticity contribution at mid-latitudes with an amplitude up to one-half that of the directly propagating wave-train. Mountains therefore play a significant role in determining the structure of the extratropical response to El Niño.

To examine the forcing of circulation anomalies, or departures from climatology, accompanying El Niño events, we attempted to simulate the difference in upper-level rotational flow between recent El Niño and La Niña winters using a spectral divergent steady-state barotropic model⁶. The large-scale flow in the upper troposphere consists mainly of horizontal motions which, outside regions of deep tropical heating, are largely non-divergent. Thus it is reasonable to diagnose global flow anomalies by modelling the vertical component of vorticity⁶, referred to here simply as 'vorticity'. On these spatial scales, the dominant source of vorticity is the conversion of the Earth's vorticity (as manifested in the Coriolis force) to flow vorticity by divergent motions, an effect analogous to the induction of electric currents in a wire loop moving through a magnetic field. In comparison to this mechanism, generation of vorticity by processes such as vertical advection, pressure-density solenoids, and tilting, can be neglected to first order. The resulting equation is generally referred to as the barotropic vorticity equation⁹.

In this study, we simulated the 200-hPa streamfunction (Ψ_{200}) differences between the 1987/88 (El Niño) and 1988/89 (La

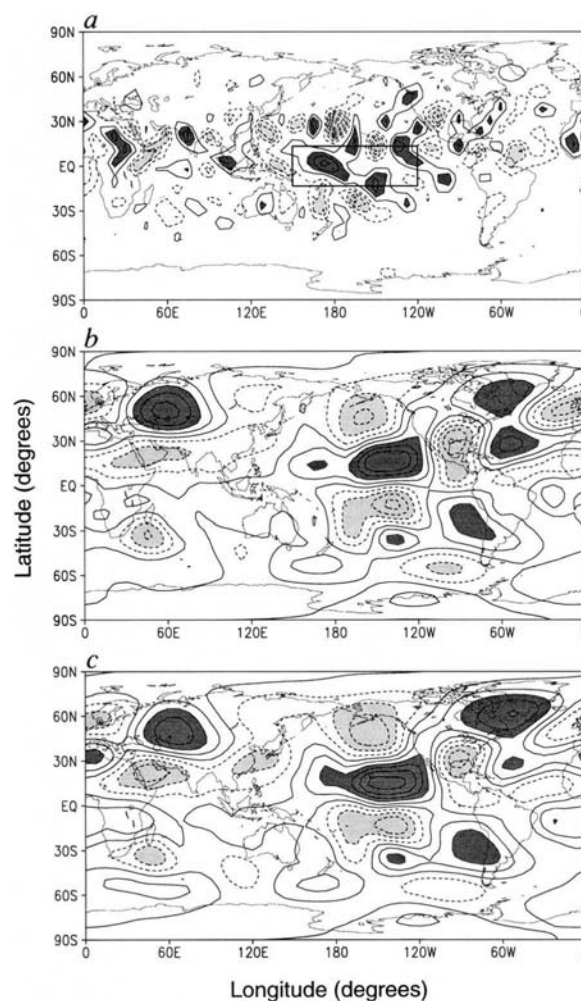


FIG. 1 Divergence and rotational flow anomalies at the 200 hPa level in the atmosphere between the winters (December, January, February) of 1987/88 and 1988/89. a, χ -derived divergence anomaly (10^{-6} s^{-1}) with zero contour suppressed, and with dark (light) shading for positive (negative) anomalies in excess of $2 \times 10^{-6} \text{ s}^{-1}$; b, simulated Ψ_{200} anomaly with the zonal-mean component removed; c, corresponding Ψ_{200} anomaly from ECMWF analyses. Contour interval is $3 \times 10^6 \text{ m}^2 \text{ s}^{-1}$, and values greater (less) than $\pm 6 \times 10^6 \text{ m}^2 \text{ s}^{-1}$ are heavily (lightly) shaded in b and c. The rectangular box in panel a defines the El Niño-related divergence anomaly used in the regional forcing experiments shown in Figs 2 and 3.

METHODS. Results displayed here are obtained from a diagnostic barotropic spectral model truncated rhomboidally at wavenumber 15, with Rayleigh damping $r = 0.2 \text{ d}^{-1}$ and biharmonic viscosity $\nu = 10^{16} \text{ m}^4 \text{ s}^{-1}$. Rotational flow anomalies are obtained by solving the following equation for ψ_a using matrix inversion:

$$\begin{aligned} \mathbf{V}_w \cdot \nabla(\zeta_c + f) + \mathbf{V}_c \cdot \nabla \zeta_a + \zeta_a \nabla \cdot \mathbf{V}_c + (r + \nu \nabla^4) \zeta_a \\ = -(\zeta_c + f) \nabla \cdot \mathbf{V}_a - \mathbf{V}_a \cdot \nabla(\zeta_c + f) - \nabla \cdot (\overline{\mathbf{V} \zeta'})_a \end{aligned}$$

Here the climatological quantity $(\cdot)_c$ is the average of that quantity for periods 1 and 2, and the anomaly $(\cdot)_a$ is the difference. f is the planetary vorticity, ζ is the relative vorticity, χ is the velocity potential, and V_x and V_w are the divergent and rotational parts of the wind field. This model solves for both the zonal-mean and eddy (zonally asymmetric) anomalies. The term on the extreme right hand side of the above equation represents the forcing by transient vorticity-flux convergence, while the first two terms represent anomalous vorticity generation by vortex-tube stretching and divergent advection of climatological vorticity. In the transient term, the overbar represents the seasonal average of the product, and the primes denote departures of V and ζ from their seasonal averages. The χ -problem is first solved for each winter season under the given model parameters.

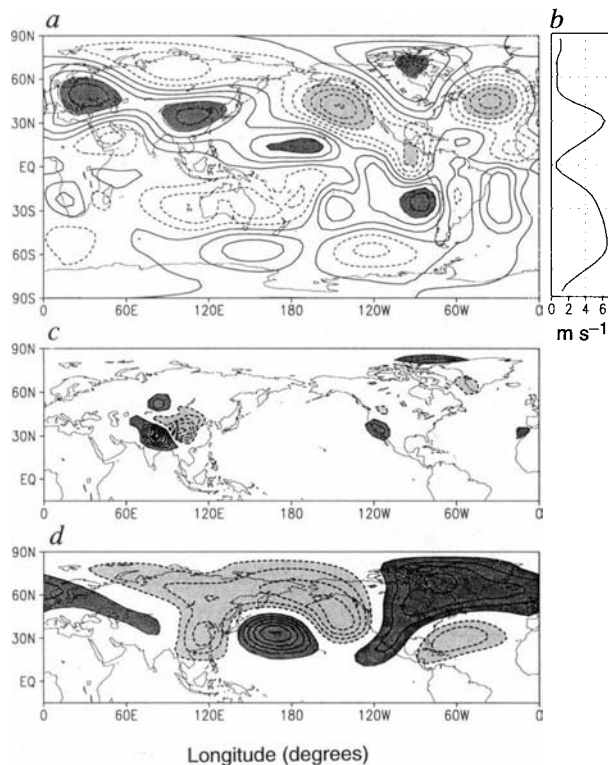


FIG. 2 The response of the model to El Niño-related divergence. *a*, Eddy response to divergence in the rectangular region marked in Fig. 1*a*, with a contour interval of $2 \times 10^6 \text{ m}^2 \text{ s}^{-1}$ and dark (light) shading for positive (negative) anomalies in excess of $6 \times 10^6 \text{ m}^2 \text{ s}^{-1}$; *b*, zonal-mean zonal wind corresponding to the solution shown in *a*; *c*, divergence (D) generated by the interaction of solutions shown in *a* and *b* with orography, with a contour interval of $2 \times 10^{-7} \text{ s}^{-1}$ and dark (light) shading for positive (negative) anomalies in excess of $2 \times 10^{-7} \text{ s}^{-1}$; *d*, eddy response to the divergence in *c*, with a contour interval of $1 \times 10^6 \text{ m}^2 \text{ s}^{-1}$ and dark (light) shading for positive (negative) anomalies in excess of $1 \times 10^6 \text{ m}^2 \text{ s}^{-1}$.

METHODS. Model solutions in *a*, *b* and *d* are obtained with model parameters as in Fig. 1. To avoid spurious rotational effects, particularly in the zonal-mean response, the global mean of the divergence anomaly is set to zero when obtaining these solutions. To ensure that the interaction with orography occurs in the far field, the orographic divergence in *c* is obtained with mid-latitude mountains only ($>25^\circ \text{N}$), although the response to divergence generated by global orography is not substantially different.

Niña) winters. In barotropic diagnosis of seasonally averaged flow, vorticity is generated by transient (sub-seasonal) fluxes of vorticity, as well as the horizontal divergence field. The horizontal divergence at 200 hPa (that is, near the tropopause) was obtained both from the European Centre for Medium Range Weather Forecasts' (ECMWF) analysed winds, and from residual diagnoses using the χ -algorithm¹⁰. This algorithm uses a variational technique to obtain a global divergence field which is consistent with the observed seasonal rotational flow and sub-seasonal vorticity transients. The vorticity transients were also calculated from the twice-daily ECMWF operational analyses on a $2.5^\circ \times 2.5^\circ$ latitude-longitude global grid. Circulation anomalies were modelled between contrasting winters using a zonally varying basic state that is the average of the two periods: when the problem is formulated in this manner, the resulting equation is automatically linear, without neglecting any terms¹¹. For example, the anomalous flux of seasonally averaged vorticity is $V_1 \zeta_1 - V_2 \zeta_2$, where subscript 1 indicates the average for season 1. This ostensibly nonlinear product can be written as $V_c \zeta_a + V_a \zeta_c$, where $(\)_a = (\)_1 - (\)_2$ and $(\)_c = [(\)_1 + (\)_2]/2$, which is linear in the anomaly fields. Thus although the steady model is posed as

a linear matrix equation, the anomaly solution is nonlinearly accurate.

Figure 1 displays the simulation produced by this model (see figure legend for the model equation). The divergence anomaly (Fig. 1*a*), obtained from the χ -algorithm using ECMWF-analysed divergence as the first guess, has a positive feature over the Equator near the dateline, in agreement with the El Niño-related anomalies in equatorial rainfall and sea surface temperature (not shown). The divergence anomalies in the subtropics and extratropics include implicitly the effects of processes such as orographic uplift and vertical motions associated with the movement of jet streams and storm tracks. The vigorous subsidence over the subtropical north central Pacific Ocean is the most significant modification of the first-guess field by the χ -algorithm. The model's Ψ_{200} response to forcing by the divergence field of Fig. 1*a* and anomalous vorticity transients (not shown) is shown in Fig. 1*b* and the corresponding observed Ψ_{200} anomaly is shown in Fig. 1*c*. A pair of anticyclones straddling the Equator east of the dateline—a tropical feature characteristic of El Niño winters—is evident both in observed and simulated anomalies. Extratropical anomalies, including the troughs centred over the Aleutian Islands and the Gulf of Mexico, are also well simulated.

Having simulated the global rotational flow anomaly, we use the diagnostic model to determine how anomalous forcing from a particular geographical region contributes to the generation of global anomalies. The geographical region of greatest interest is of course the central/eastern tropical Pacific (the rectangular box in Fig. 1*a*) where the El Niño-related heating is strongest. Figure 2*a*, *b* shows the model response when forced exclusively by the divergence in the rectangular box in Fig. 1*a*. The response shown here is quite similar to the model response when forced by the entire tropical strip, or, for that matter, by the idealized source used in Fig. 4.

An interesting feature of Fig. 2*a*, *b* is that both the zonal-mean and eddy winds have large amplitude in mountainous regions. Furthermore, as the largest mountains are in mid-latitudes and thus in the far field of the El Niño-related divergence, we can assume that in the vicinity of the mountains the tropically forced wind perturbations blow in the same direction at all levels, so that only their amplitude varies with height¹². In that case, the divergence D due to orographic uplift can be estimated in an

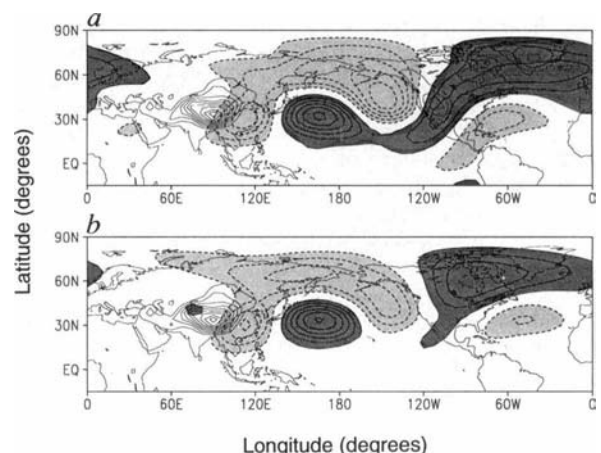


FIG. 3 Orographic modulation of the response to El Niño-related divergence as simulated by a barotropic model which includes orographic stretching in the operator. *a*, Modulation by extratropical ($>25^\circ \text{N}$) mountains; *b*, modulation by the extratropical eastern hemisphere ($\leq 180^\circ \text{E}$) orography. Contour interval is $1 \times 10^6 \text{ m}^2 \text{ s}^{-1}$ and dark (light) shading is for positive (negative) anomalies in excess of $1 \times 10^6 \text{ m}^2 \text{ s}^{-1}$. Thin solid lines contour the orography greater than 500 m with an interval of 500 m.

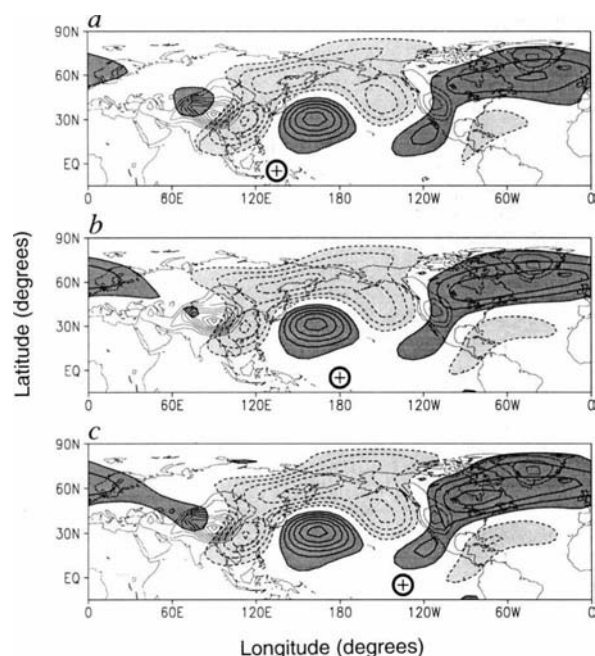


FIG. 4 Modulation of the response of the model to an idealized tropical divergence anomaly placed at 5° S and longitudinally at 135° E (a), 180° (b), and 135° W (c) by extratropical ($>25^{\circ}$ N) mountains. Contour interval and orographic contouring as in Fig. 3. Divergence anomalies are ellipses centred on the “+” sign in each panel, with zonal and meridional extent of 60° and 20° , respectively, and with maximum amplitude of $6 \times 10^{-6} \text{ s}^{-1}$.

off-line calculation by blowing scaled-down winds over the mountains: $D = \alpha \mathbf{V}_{\psi} \cdot \nabla h / (H_0 - h)$. Here H_0 ($=8 \text{ km}$) is the scale height of the atmosphere, h is the height of the mountains, and $\mathbf{V}_{\psi}$ is the tropically forced rotational wind anomaly, scaled down by a factor $\alpha = 0.3$. Figure 2c shows the orographic divergence D , and Fig. 2d shows the model's response to D . The response to D contains large-scale features in the Pacific/North American sector (for example, the low over the Gulf of Alaska and the ridge extending across North America from Baja California to Greenland) that could substantially modify the directly forced extratropical wave pattern. The extratropical geopotential height anomalies associated with this streamfunction perturbation exceed 40 m , while the corresponding zonal wind anomaly exceeds 5 m s^{-1} in the central Pacific, where it overlaps the eastern end of the East Asian jet. Thus the zonal wind anomaly acts to extend the East Asian jet southeastwards, as is typical in El Niño winters. The strength of the orographically induced rotational flow is about one-third of the flow anomaly forced directly from the tropics, and also about one-third of the observed anomalies (Fig. 1c).

The model presented thus far has been a completely literal representation of our physical intuition: tropical heating excites the primary wave pattern, which impinges on the mountains, generating, in a separate calculation, a secondary response which has no further interaction with orography. Although well motivated, this model has an *ad hoc* character which is both undesirable and unnecessary. A more general model can easily be constructed by including the orographic divergence D in the linear operator rather than treating it as external forcing. When orography is represented explicitly in the time-mean divergent barotropic equation, the resulting term $S = -(\nabla^2 \psi + f) \alpha \mathbf{V}_{\psi} \cdot \nabla h / (H_0 - h)$, often referred to as the orographic stretching term, is quadratic in ψ . But as the anomaly model is formed by taking the difference between the time-mean equations for the two seasons, the anomalous orographic stretching term is linear¹¹ in ψ_a . With this term in the operator, the model can be written as a single equation which includes both the primary forcing by El

Niño-related divergence and the secondary effect of orographic modulation.

Figure 3a shows the orographic modulation as simulated by the new model. We display the difference between the solution for $\alpha = 0$ (without orographic modulation) and the solution for $\alpha = 0.3$. This difference map is remarkably similar to the secondary wave pattern of Fig. 2d. The similarity suggests that the wave pattern of Fig. 2d has little further interaction with orography, although the new model does allow for such interactions. Further analysis reveals that the anomaly pattern of Fig. 3a is generated primarily by the interaction of the zonally asymmetric flow with orography. Figure 3b shows the contributions of the eastern hemisphere orography to the pattern in Fig. 3a. This figure shows the Himalayan–Tibetan complex to be the major locus of orographic interaction. This result is somewhat surprising, given that the primary wave-train of Fig. 2a appears to impinge more directly on the North American land mass.

Although there is no guarantee that a more complex multi-level model will reproduce the orographic interaction described here, there are reasons for believing that the response will be similar. In general circulation model experiments, the response in the Pacific/North American sector to tropical heating anomalies is relatively insensitive to the longitudinal position of the heating anomaly⁵. If the primary wave pattern in the Himalayan–Tibetan region is likewise insensitive to source longitude, the orographic modulation will be as well. As a preliminary test of this sensitivity, we performed an experiment with our simple model in which an idealized tropical divergence anomaly was placed in the equatorial Pacific at three different longitudes. From results shown in Fig. 4, it is clear that even when the divergence anomaly shifts across the Pacific from 135° E to 135° W, the orographic interaction generates anomalies which are qualitatively quite similar. The insensitivity to heat source location is inherited from the similarity of the three primary wave patterns in the Himalayan–Tibetan region. Additional analysis of the anticyclonic cells over Eastern Europe and China in the primary wave pattern (Fig. 2a) indicates that the cell over China is an upstream ‘tail’¹³ generated by the large meridional vorticity gradients of the Asian jet, whereas the Eastern European cell results from downstream wave propagation through the zonally varying climatological flow.

In a linear model such as ours, an insensitivity in the response can arise for two reasons: either the forcing functions have similarities, or the linear operator representing the basic state has some preferred patterns of response. We have examined the structures of the vorticity sources for the three cases in Fig. 4, and found them to be quite different, which would suggest the second explanation. Preferred response patterns have sometimes been explained in terms of unstable normal modes of the operator¹⁴. But because the results presented here were obtained as the steady response to external forcing in the presence of vorticity dissipation, we believe that the preferred response patterns in our case can be better understood in terms of the refractive properties of the zonally varying climatological flow¹³. □

Received 31 July; accepted 9 November 1995.

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ACKNOWLEDGEMENTS. We thank M. Cai for help in model validation, and F. Baer for his encouragement and support. This work was supported by the US Department of Energy, the US NSF, and the National Oceanic and Atmospheric Administration.

Mantle dynamics and the heat flow into the Earth's continents

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REDUCED continental heat flow, defined as the measured heat flow minus that due to radiogenic sources in the upper crust, initially decreases with time after the last thermotectonic event in a region¹, but then tends to stabilize at a roughly constant value, which is higher than would be predicted by a conductive cooling model (Fig. 1). Here we argue that this behaviour results from the influence that continents have on mantle heat loss. The finite thermal conductivity of continental crust and the fact that it is not recycled into the mantle on a large scale, as is oceanic crust, allows stable continental blocks to limit the local heat flux out of the mantle. Numerical models that allow blocks of crust to form over a mantle layer demonstrate how thermal perturbations associated with mantle convection penetrate into continents, causing lateral temperature variations at their base that parallel those in the mantle just below. This tends to establish a constant flux of heat from the mantle into the continents, which accounts for the trend in reduced heat flow.

The behaviour shown in Fig. 1 has previously been interpreted as evidence of additional heat input into the base of old continental lithosphere due to a secondary scale of convection—that is, one of a smaller scale than that associated directly with the formation of tectonic plates at mid-ocean ridges and their reincorporation into the interior of the mantle at subduction zones^{2–4}. Although the elevated value of reduced heat flow is due in part to lower-crustal heat sources, regions where lower-crustal heat production can be estimated suggest that a mantle component still accounts for up to 50% of the observed heat flow⁵. This allows mantle causes to remain attractive for explaining the trend shown in Fig. 1. The argument that small-scale mantle convection is responsible for it is the same as that used to explain the flattening of the sea-floor depth versus age curve in old ocean basins⁶; the lower lithosphere is assumed to behave like a thermal boundary layer that thickens with time up to a point at which its negative density overcomes its viscosity allowing portions to drip off into the asthenosphere thereby maintaining a constant lithospheric thickness. Assuming near-constant temperature at the top of the mantle lithosphere and in the asthenosphere, this leads to constant mantle heat flux for litho-

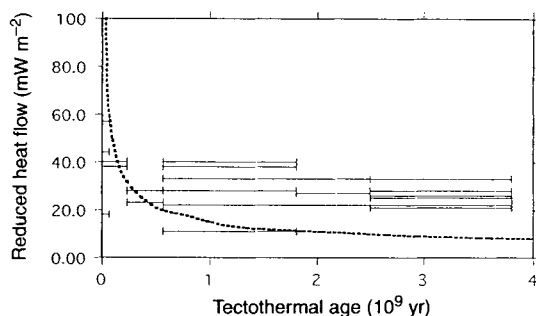


FIG. 1 Reduced continental heat flow in various heat-flow provinces plotted versus time since the last thermotectonic event in the province, along with a curve of the expected heat flow if the lithosphere cooled conductively with age. Reduced heat-flow values are plotted as lines spanning the age range of a province¹. The lowest heat-flow value has the largest error, $\pm 73\%$. The average error for all other provinces with a thermotectonic age greater than 65 Myr is $\pm 14\%$. The conductive curve assumes mantle thermal properties from ref. 19.

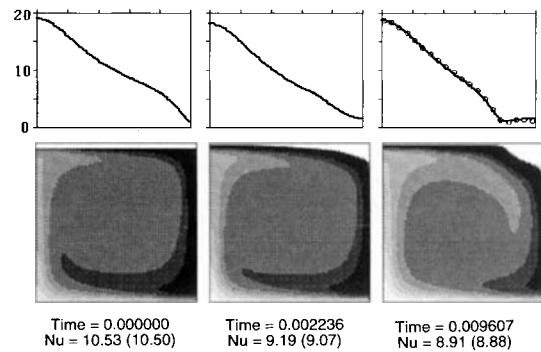


FIG. 2 Non-dimensional surface heat flux (top row) and density field (bottom row) evolution from an isoviscous model ($Ra = 10^3$, $Ra_c/Ra = 2$, and $ICD = 0.02d$, where d is the system depth). The lightest tone in grey-scale images represents lowest-density material, that is, a model analogue to chemically light crust; the darkest tone represents high-density material (mantle lithosphere). An 80×80 horizontally uniform and vertically non-uniform mesh was employed (40 elements across the top 20% of the domain). The non-dimensional temperature at the top and bottom of the domain is 0 and 1, respectively, and both surfaces are free slip while sides are reflecting. Non-dimensional time and the Nusselt number, Nu , are shown below each density frame. The Nusselt number from an equivalent parameter run on a 52×52 mesh with 20 elements across the top 20% of the domain is included in parenthesis as an indication of solution resolution. The heat flux at a time of 0.024242 is plotted on the last graph by open circles to show that it has reached a slowly varying state. The equilibrium heat flux was not qualitatively altered by inclusion of temperature-dependent crustal and mantle viscosities^{9,10}.

sphere older than 10^8 years, the time at which small-scale convection is predicted to set in⁶. Whether this mechanism operates below oceans is well debated^{7,8}. Our intent is not to question its viability, but to present an alternative explanation for the reduced heat flow trend that requires minimal assumptions.

Figure 2 shows results from a mantle convection model with a near surface thermal/chemical boundary layer. The model, more fully described elsewhere^{9,10}, consists of coupled mass-, momentum-, energy- and composition-conservation equations which are solved via a finite-element formulation^{11,12}. Initially a thin layer of chemically light material (mimicking crust) is imposed within the upper thermal boundary layer of chemically dense, thermally convecting material (which is mimicking mantle). The initial thermal field is obtained by solving model equations with only mantle present. Evolution is determined by three factors: (1) the thermal Rayleigh number, Ra , which governs the vigour of thermal convection in the mantle; (2) the ratio of the chemical to the thermal Rayleigh number, Ra_c/Ra , which is a measure of chemical density variations relative to thermal; and (3) the initial depth of the crustal layer, ICD . Convective overturn of the mantle causes crust to thicken above a zone of thermal downflow. As it does, local surface heat flux tends toward a spatially constant value. Given that crustal heat flow is largely conductive and that no crustal heat sources are present, this implies that mantle heat flux below the crustal accumulation is also tending towards a constant value. This tendency is robust under a range of parameter variations^{9,10}.

Model results are simply understood through a consideration of how a convecting layer exchanges heat with its surroundings. The thermal coupling condition between a convecting fluid and a layer of thickness h that bounds it from above can be expressed in terms of a Biot number

$$Bi = (\delta/h)(\kappa_c/\kappa_i)$$

where κ_i and κ_c are the thermal diffusivities of the convecting fluid and the bounding material, respectively, and δ is the thick-

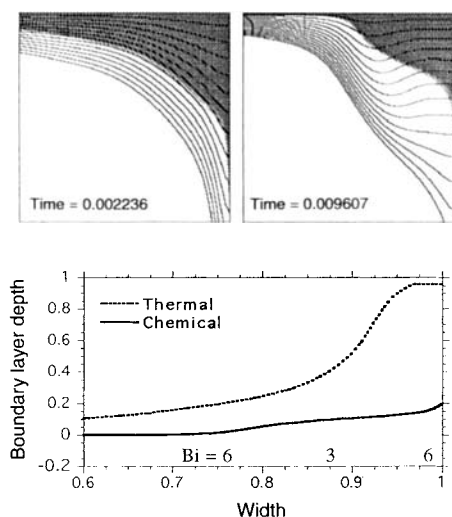


FIG. 3 Temperature isotherms over composition shade plots (top row) from the upper right portion of the model in Fig. 2 along with a plot of chemical boundary layer, CBL, and upper thermal boundary layer, TBL, depth at time 0.009607 (bottom graph). The physical dimension of the vertically stretched images is $0.5d$ width $\times$ $0.2d$ depth. The 15 equally spaced isotherms span the cold half of the temperature field and dark shade represents crust. The depth to the 0.5 isotherm is used as a gauge of TBL depth. Similarly, CBL depth is that to the mean composition field value (this is within two elements of pure crustal or mantle component for most of the domain). The Biot number at three points across the crustal accumulation is shown in the lower graph (CBL depth is equated with h , and TBL minus CBL depth with δ , in the expression for the Biot number given in the text). Notice in the images how formation of the crustal accumulation induces a strong lateral temperature gradient in the portion of the TBL near its edge. For similar models with a larger Ra or a larger aspect ratio, this triggered secondary TBL instabilities near the edge of the accumulation^{9,10}.

ness of the upper thermal boundary layer in the convecting fluid^{13,14}. For Bi approaching infinity, constant temperature prevails at the surface of the convecting layer; for Bi approaching zero, constant heat flux prevails. For the model of Fig. 2, Bi tends to a value of order unity in regions of crustal accumulation and is infinite elsewhere (Fig. 3). For regions of infinite Bi thermal perturbations that might be generated at the boundary of the convecting mantle, owing to heat loss from within, are rapidly 'washed away' because of the large thermal diffusivity of bounding material which, for oceanic regions, is in effect water. For regions of low Bi , thermal perturbations penetrate into bounding material leading to a lateral temperature gradient at its base that mimics that in the convecting fluid below. By virtue of the fact that they are not reworked into the convecting mantle on the scale that oceanic crust is, the continents act as thermal bounding material for the mantle which, given the comparable diffusivities of distinct rock types, leads to an equilibrium thermal condition of near constant mantle heat flux at the base of continents.

The equilibrium condition noted above can also lead to near-equal heat flux into stable continental blocks that form in spatially separated regions of the mantle (Fig. 4). Density undulations in the model lithosphere of Fig. 4 result from small-scale thermal boundary layer instabilities that preferentially form near the edges of crustal accumulations. For the early evolution of the model, these instabilities circulate within the pre-existing large-scale thermal cells in the mantle. In time they can also alter the large-scale convective structure. The cold boundary layer instability seen forming at the edge of the left-hand crustal accumulation at time 0.011011 (Fig. 4) transverse the system depth inducing a flow reversal below the crustal mass which causes the mass to drift to the right. As it does, it retains its thermal structure owing to the slowness of thermal diffusion relative to the

advective timescale of the initial drift. This retention maintains a constant heat flux out the accumulation despite the fact that it drifts over warmer mantle. During this time an equilibrium thermal condition does not hold at its base. As it can carry its thermal structure, the drifting crustal mass also induces large lateral temperature gradients ahead of itself which favour the formation of cold mantle downwellings (the initial phase of this can be seen in the last density frame of Fig. 4; the cold thermal boundary layer instability forming at the edge of the crustal mass in that frame develops into a cell defining downwelling). This allows the heat flux out of the left-hand crustal mass in Fig. 4 to stay constant even after it settles over a new position above the mantle. The implication is that for drifting continents reduced heat flow can remain constant because the continents can carry their thermal structure and because they have the potential to modulate the thermal mantle structure below themselves. Of course a long-lived thermal upflow below a continent, a model analogue of which is the left-most hot jet in the last frame of Fig. 4, can still short-circuit the constant-flux tendency, but such an upflow and the effects that it induces would be defined as an event that should locally reset the thermotectonic age of a province (Fig. 1).

The explanation offered here for reduced heat flow implies that the effective surface thermal condition seen by the Earth's mantle is spatially and temporally variable. This suggestion is not new and has directly, or indirectly, been applied to the idea that continental drift is driven by the insulating, or local heating, effects of continents themselves^{15,18}. Apart from this, however, it has received little direct attention and the wealth of models constructed over the years to help better understand mantle convection have dominantly assumed constant-temperature boundaries as appropriate or adequate at low order. Theoretical or experimental studies of convecting systems with spatially, or spatially and temporally, variable thermal boundary conditions

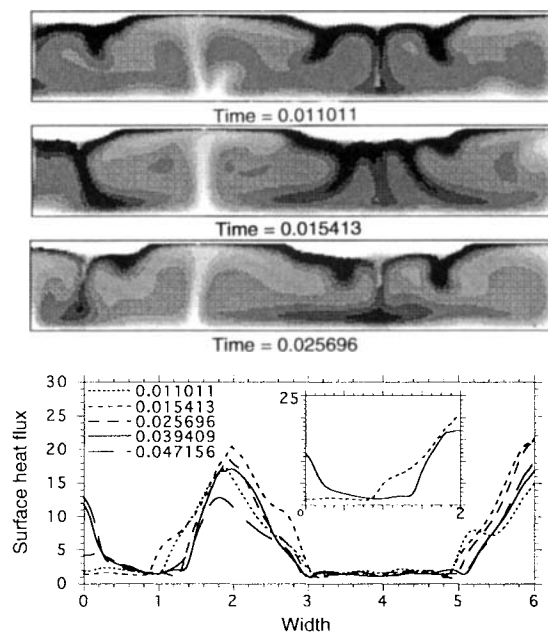


FIG. 4 Density field (upper images) and heat flux (lower graph) evolution from a 6×1 aspect-ratio model ($Ra = 10^5$, $Ra_c/Ra = 2$ and $ICD = 0.04d$). The inset in the lower graph shows the heat flux across the left third of the model domain. A 200×50 horizontally uniform and vertically non-uniform mesh was employed (20 elements across the top 20% of the domain). The initial thermal field had three equal-dimension convection cells filling the domain, with cell-defining thermal downwellings centred at domain width positions of 0.0 and 4.0. The left-hand crustal mass spanned a width from 0.0 to 0.9 from the time of its formation to time 0.02; at time 0.05, it came to span a width from 0.5 to 1.4 with a cell-defining thermal downwelling centred below it.

have, on the other hand, remained relatively scarce. The essence of our primary argument has been that if one accepts that the Earth's mantle is such a system, then an understanding of the physics behind the observation contained in Fig. 1 follows with few assumptions in between. □

Received 19 July; accepted 6 November 1995.

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ACKNOWLEDGEMENTS. Computing was carried out at SDSC, San Diego. We thank M. Gurnis for a review; S. D. King, A. Raefsky and B. H. Hager for the ConMan finite element code; and NCSA, Illinois for visualization packages. This work was supported by NASA.

Cycliophora is a new phylum with affinities to Entoprocta and Ectoprocta

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THE mouthparts of the Norway lobster *Nephrops* are colonized by an acelomate metazoan, *Symbion pandora* gen. et sp. nov. Sessile stages continually produce inner buds replacing feeding structures. They also produce one of three motile stages: (1) larvae containing new feeding stages, (2) dwarf males, which settle on feeding stages, or (3) females, which settle onto lobster mouthparts, and eventually degenerate, giving rise to dispersive larvae. All motile stages are short-lived, and do not feed. The structure and function of the cilia suggest a phylogenetic position in Protostomia, while some aspects of inner budding and brooding of larvae are similar to those of Entoprocta and Ectoprocta. The dispersive larva possesses a mesodermal supporting chordoid structure, otherwise absent in protostomial larvae. We believe that all the above features of this previously undescribed species warrant the recognition of a new phylum with affinities to Ectoprocta and Entoprocta.

Cycliophora, new phylum

Diagnosis. Acelomate, marine metazoans with bilateral symmetry and differentiated cuticle. Compound cilia engaged in filter feeding working as a downstream-collecting system. Sessile, solitary feeding stages with multiciliated cells in the gut, anus just behind a ciliated mouth ring, and internal budding with extensive regeneration of feeding apparatus. Feeding stages alternating with brief, non-feeding, free-swimming stages. Brooding of asexual larva (Pandora larva), male and female. Females brooding chordoid larva with a mesodermal, ventral rod of plate-like muscle cells (chordoid structure) and a pair of protonephridia with multiciliated terminal.

Etymology. Cyclion and phoros are Greek for 'a small wheel' and 'carrying' referring to the circular mouth ring.

Eucycliophora, new class

Diagnosis. Same as the phylum.

Etymology. Eu is Greek for 'well', referring to the splendid symmetrical mouth ring.

Symbiida, new order

Diagnosis. Same as the phylum with metagenesis in the life cycle.

Symbiidae, new family

Diagnosis. Same as the phylum.

Symbion gen. nov.

Diagnosis. Same as the phylum.

Type species. *Symbion pandora*, new species by designation (Figs 1–4).

Etymology. Syn and bios are Greek for '(together) with' and 'living', referring to the intimate life with the crustacean host; masculine gender.

Symbion pandora sp. nov.

Diagnosis. Same as the phylum.

Etymology. The feeding stage which contains both an inner bud and a Pandora larva with a miniature feeding stage inside (Fig. 4, stage 9) reminds one of Pandora's box.

Type material. The holotype is a feeding stage to which the allotype, a mature dwarf male, is attached (Fig. 1). Both are as total preparations on a microslide. This slide, together with several hundred paratypes, are deposited in the Zoological Museum of Copenhagen (ZMUC CYC-0001–CYC-0300), Denmark. The holotype and allotype occurred on the mouth appendages of *Nephrops norvegicus*, taken off Frederikshavn, North Kattegat, Denmark (57°26' N, 10°42' W), at a depth of 20–40 m, 19 August 1991. Additional paratypes were collected from *N. norvegicus* taken at Frederikshavn, Denmark; Gullmarfjord, Sweden; and Kaldbak Fjord, Faroe Islands, North Atlantic Ocean. Part of this material is placed in ZMUC and in the National Museum of Natural History, Smithsonian Institution (USNM), Washington DC, USA.

Holotype. Length, 347 µm; width, 113 µm. Body consists of buccal funnel, trunk, and stalk with disc attached to a host seta. The bell-shaped buccal funnel is constricted at the base and opens anteriorly to form a circular mouth circumscribed by a ciliated mouth ring. This ring is extended in the normal feeding position. The funnel is bent over the anus towards the host's seta. The ovoid trunk is borne on a short (63 µm) stalk which expands to an attachment disc (73 µm across). The funnel, trunk, stalk and attachment disc are lined by a cuticle exhibiting a characteristic pentagonal or hexagonal sculpturing. The stalk and attachment disc consist exclusively of cuticular material.

The buccal funnel narrows into a short S-shaped oesophagus, which continues into the U-shaped alimentary tract. The descending part of the alimentary tract is an enlarged stomach. The stomach wall consists of large, ciliated cells. The stomach is filled with a cluster of granular, secretory cells without cilia, reducing the stomach lumen to lacunar spaces. The ascending part of the alimentary tract consists of a ciliated intestine and a short rectum. In the trunk, the space between the epidermal layers is totally occupied by large vacuolated mesenchyme cells. There is no coelom. The holotype contains a single internal bud which has developed a new mouth ring and ciliated gut. This has pushed the old digestive system forward. Two clusters of cells are situated lateral to the inner bud. We interpret these as early embryos, supported by observations on paratypes.

Allotype (dwarf male). The allotype is a mature male attached to the trunk of the holotype. Total length, 84 µm; maximum width, 42 µm. The male has an ovoid trunk (67 µm), a short stalk (17 µm) and a large attachment disc (27 µm across). Buccal

funnel and anus are absent in the male. The sculpture of the cuticle is the same as that of the holotype, but the polygonal markings are finer. The stalk and disc consist only of cuticular material.

An alimentary tract is absent. Distal to the adhesive disc, two large compartments lined by very thin cuticle are present. Each compartment contains a body of differentiated cells surrounded by a thin cuticle, except in two V-shaped areas with ciliated epithelia. Each body contains different stages of spermatids or spermatozoa and a tubular, cuticular structure, here interpreted as a penis. A cluster of undifferentiated cells is situated near the stalk. The rest of the interior is filled with vacuolated mesenchyme cells.

Paratypes. The mouth ring consists of alternating multiciliated and myoepithelial cells. The latter form two sphincters (peripheral and central) involved in closing the mouth. In live specimens the compound cilia of the mouth ring were observed to collect particles with a downstream collecting system. The lining of the buccal funnel consists of multiciliated cells with compound cilia. The anus is a distinct transverse fissure (about 25 μm long) on a small elevation on the trunk close to the buccal funnel. Transmission electron microscopy (TEM) studies show the 1–2- μm -thick cuticle to be composed of a very thin trilaminar epicuticle and a thick fibrillar procuticle. TEM of the alimentary tract shows multiciliated cells with separate cilia of the digestive tract, a cuticular lining of the rectum and a tuft of long cilia at the entrance to the stomach. In living specimens these long cilia move in an undulatory way and the contents of the intestine

rotates. Two long muscle fibres connect the base of the buccal funnel on the anal side with the opposite side of the trunk. These muscles are probably responsible for the only movement observed: a nodding with the buccal funnel. Some muscles are cross-striated, others obliquely striated. A ganglion (the brain) is situated between the oesophagus and the rectum in the trunk region. The position of the brain means that the buccal funnel is not homologous with a true head. The two clusters of cells situated lateral to the inner bud, as described in the holotype, might be early embryos. This is supported by observations of a series of paratypes with intermediate stages leading to a single, fully developed embryo (Fig. 4, stages 7–12–15–16). The development of an inner bud is shown in Fig. 4, stages 4–7. In stages without an internal bud, the alimentary tract extends to the stalk. As the internal bud develops further inside the feeding stage, the old digestive system totally disintegrates and the buccal funnel is finally cast off (Fig. 4, stage 8). The new buccal funnel and alimentary tract formed from the inner bud subsequently emerge and start feeding. Inner budding is repeated several times in the lifetime of an individual.

The male cuticle is ultrastructurally similar, but thinner than that of the holotype. Male paratypes with one and three compartments were also observed. In the cellular parts of the compartments a ring of well-developed, cross-striated muscles underlies the epidermis. Other cross-striated muscles attach to the base of the penis. A large two-lobed ganglion is located in the cellular body inside each compartment. Continuing TEM analysis of males attached to feeding stages could suggest that

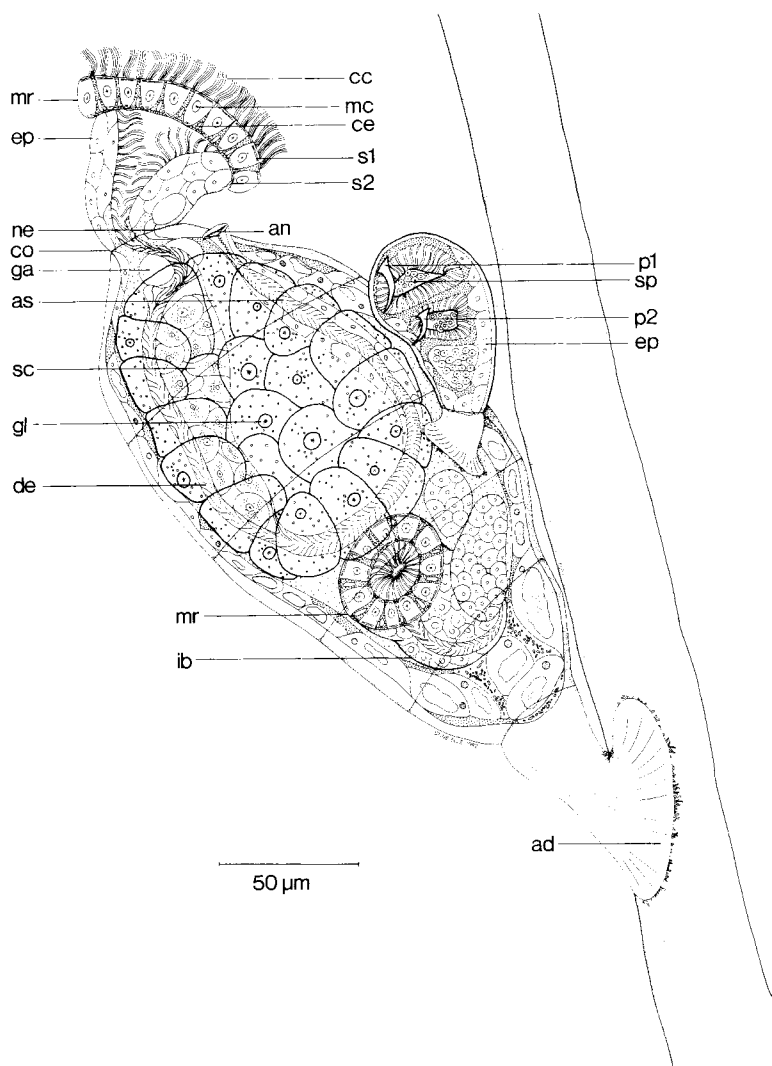


FIG. 1. *Symbion pandora*, new species, holotype and allotype (ZMUC CYC-0001). The holotype is an asexual feeding stage, attached to a seta of a mouth limb from *Nephrops norvegicus*. The allotype is a mature male, a dwarf male attached to the holotypic feeding stage. The specimens were relaxed with MgCl_2 before fixation in formalin. ad, adhesive disc; an, anus; as, ascending branch of the digestive system; cc, compound cilia; ce, ciliated epidermis; co, constriction (or 'neck'); de, descending branch of digestive system; ep, epidermis; ga, ganglion; gl, gut lining cell; ib, inner bud; mc, myoepithelial cell; mr, mouth ring; ne, nerve; p1, penis 1; p2, penis 2; sc, stomach cells; sp, sperm; s1, sphincter 1; s2, sphincter 2.

each compartment actually represents a brood chamber for an extremely reduced secondary male formed by inner budding.

The life cycle proposed in Fig. 4 is based on: (1) live studies of different free-swimming individuals liberated from feeding stages of *S. pandora*, (2) TEM of fully developed stages still inside the feeding stages and (3) the temporal occurrence of different stages of *S. pandora* relative to the phases of the host's moulting cycle; for example, chordoid larvae, males and females are numerous on *Nephrops* close to moulting, and absent on those newly moulted.

All ciliated epithelia of *S. pandora* consist of multiciliated cells as in spiralian larvae and rotifers¹. In the feeding stage, the bands of compound cilia work as a downstream collecting system², indicating that *S. pandora* belongs to protostomians³. The attached dwarf male of *S. pandora* reminds one of some rotifers. This similarity is apparently superficial, as rotifers do not have a true cuticle⁴, lack regenerative powers and have dimorphic spermatozoa^{5,6}, which have not been observed in *S. pandora*. The cuticle of *S. pandora* is similar to that found in some Nematoda⁷ and Gastrotricha⁸. Continuing TEM analysis of the chordoid larva shows that the mesodermal chordoid structure

resembles similar tissue found in some free-living Gastrotricha⁹, such as *Chordodasys*. There is no evidence for any homology with the dorsal chorda of Chordata, because the chordoid structure in *S. pandora* is situated ventrally. Internal budding, a salient feature of *S. pandora*, is absent in all Aschelminthes, which in general lack powers of regeneration. Nevertheless, Manylov¹⁰ recently described regeneration in the gastrotrich *Turbanella*. We would advocate that a relationship exists with the phyla Entoprocta and Ectoprocta united by Nielsen^{3,11} in the superphylum Bryozoa. The fact that all three groups share a U-shaped gut is of minor importance, as this is common among other sessile taxa. Although nephridia have never been reported in Ectoprocta, both Entoprocta and the chordoid larva of *S. pandora* possess protonephridia with multiciliated terminal cells. Budding is characteristic of all Entoprocta and Ectoprocta. Acceleration of the development of feeding structures occurs in the larvae of freshwater ectoprocts in the order Phylactolaemata^{12,13} and in certain Entoprocta such as *Loxosomella vivipara* and *Loxosoma jaegersteni*¹¹. This is reminiscent of the advanced development

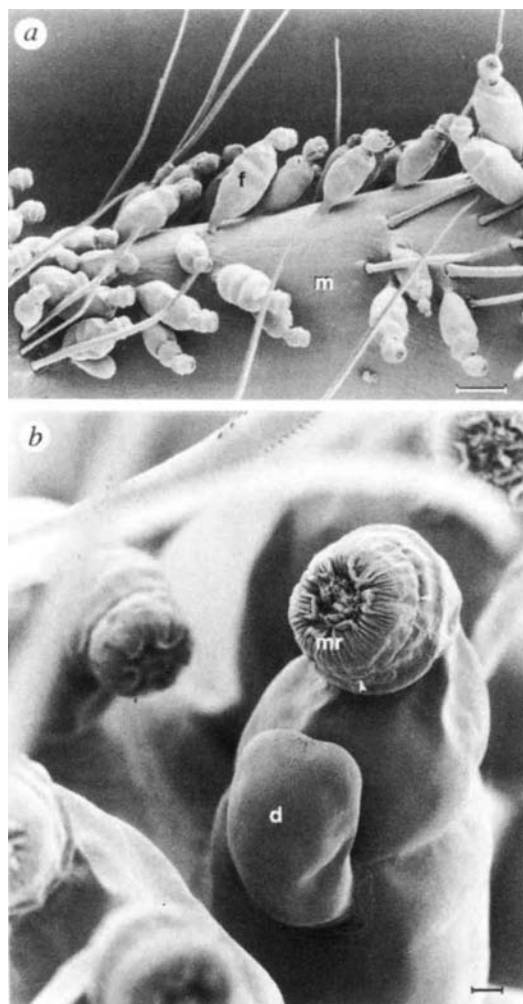


FIG. 2 *Symbion pandora*, new species. SEM of paratypes from Kaldbak Fjord, Faroe Islands. *a*, Overview of *Nephrops* mouthpart (m) with numerous feeding stages (f). Scale bar, 100 μ m. *b*, Detail of a feeding stage with a closed mouth ring (mr); arrowheads point to basal border of the mouth ring. Compare with Fig. 1 illustrating the open mouth ring in feeding position. One dwarf male (d) is attached to the trunk of the feeding stage. Often several males are attached to a single feeding stage. Scale bar, 10 μ m.

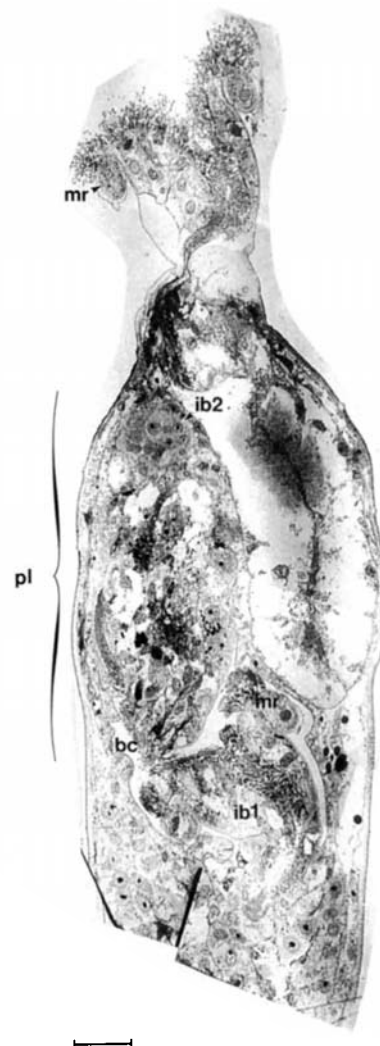
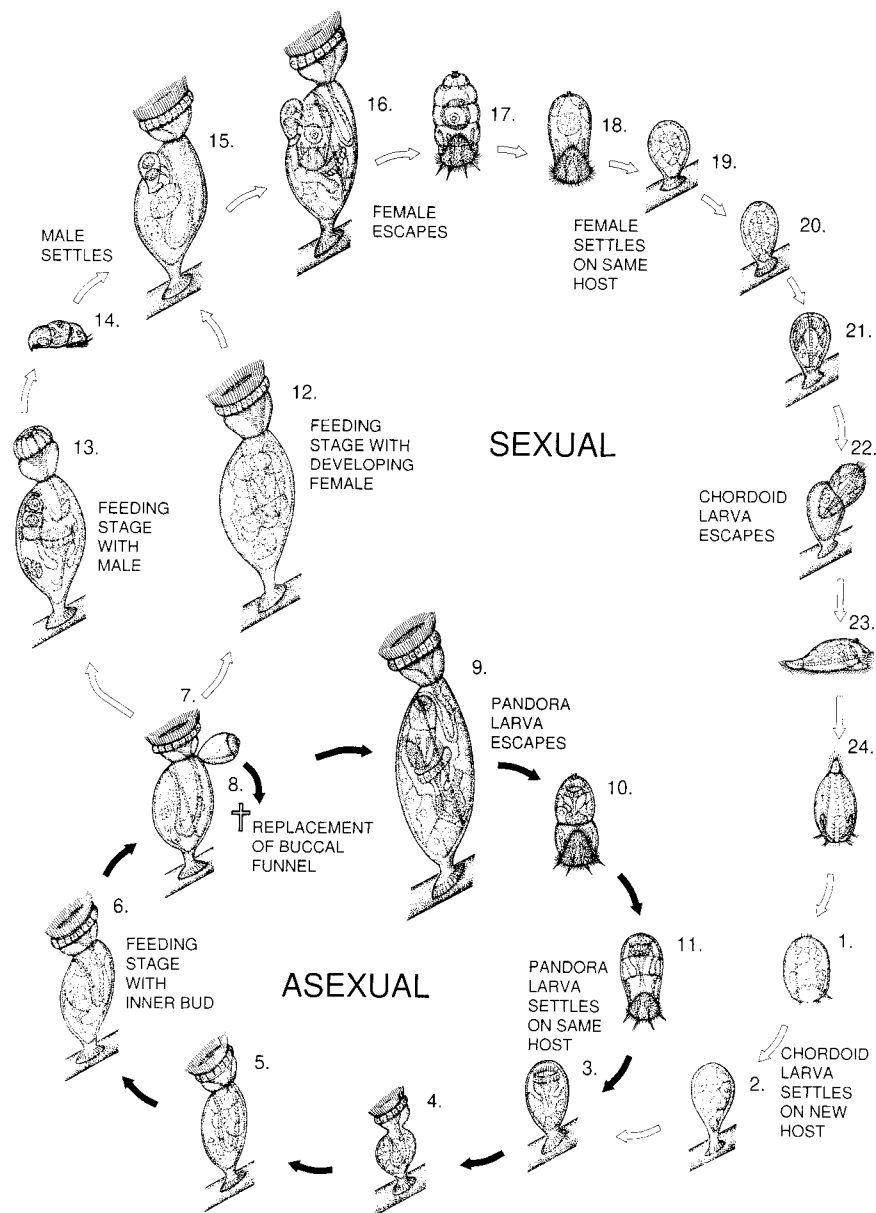


FIG. 3 *Symbion pandora*, new species. TEM mosaic micrograph of paratype from Kaldbak Fjord, Faroe Islands. A longitudinal section of a feeding stage with a Pandora larva (pl) and an inner bud (ib1). The larva and the bud are developed in a brood chamber (bc), lined with cuticle. The inner bud has differentiated into a distinct mouth ring (mr) and ciliated buccal funnel, like those of the maternal feeding stage. Inside the Pandora larva, another inner bud (ib2) later develops into a new feeding stage. Scale bar, 20 μ m.

FIG. 4 Hypothetical life cycle of *Symbion pandora* new species. A free-swimming chordoid larva with internal buds settles on the mouth appendages of the crustacean host (1–2). The chordoid larval body degenerates. Asexual life cycle: The bud develops into a small feeding stage (3). When the mouth funnel emerges (4) feeding begins. Growth of the feeding stage is followed by successive inner buddings with total regeneration and replacement of buccal funnel, alimentary tract and nervous system (5–8). Reaching a certain size (maturity), the feeding stage develops asexual Pandora larva in a brood chamber (9). A small new feeding stage develops through budding inside the Pandora larva, which is still inside the brood chamber (9). At the same time another inner bud is developed (9), also inside the maternal feeding stage; this bud eventually differentiates into a new buccal funnel (9). The Pandora larva (10–11) escapes, settles and repeats growth as described (3–8), finally producing a new Pandora larva (10). This asexual part of the life cycle explains the large populations of feeding stages with no mature sexual stages found on many lobsters. Sexual life cycle: On lobsters near to the end of a moult cycle sexual mature stages are found. Some feeding stages develop one dwarf male (13) in a manner similar to the development of the Pandora larva. The dwarf male escapes (14), now filled with mature sperm. It settles on a feeding stage with a developing female inside (15). The female contains a large oocyte (16), and escapes with the posterior end first. The female settles on the mouth appendages of the crustacean host. It attaches with the anterior, ventrally oriented cilia, secreting the contents of numerous adhesive glands. The chordoid larva develops from the zygote inside the female (17–18). The female dies, leaving a cuticular shell with a developing embryo (19) differentiating into a larva with a mesodermal chordoid structure and a pair of protonephridia (20–21). This chordoid larva hatches (22); it is lecithotrophic, equipped with extensive locomotory ciliation, a pair of dorsal ciliated organs and numerous glands of different types (23, lateral view; 24, ventral view). The chordoid larva settles (1–2), and metamorphosis leads to the sessile stage (2) with a developing bud. This bud develops into a feeding stage (3) initiating the asexual life cycle.



of the mouth ring and gut of the feeding stage within the Pandora larva. There are also some similarities in brooding comparing *S. pandora* with the phylactolaemate ectoprocts. Furthermore, among the Protostomia, Entoprocta and Ectoprocta are the only groups in which the larval brain disappears³ at metamorphosis. The brain of *S. pandora* also disappears in all the free-swimming stages at settlement. Inner buds generate new individuals and

new nervous systems in the chordoid larva, the Pandora larva and perhaps the male. The colonies of Ectoprocta also arise by budding³. We propose that the Cyclophora represents a new phylum with the following autapomorphies: dwarf males, metagenesis in the life cycle, a chordoid larva and a ciliated mouth ring. The sister group of Cyclophora might be Entoprocta or superphylum Bryozoa. ■

Received 31 July; accepted 2 October 1995.

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ACKNOWLEDGEMENTS. Our sincere thanks to T. Fenchel (Marine Biological Laboratory, Elsinore, Denmark), who discovered the new epizoon. We thank C. Nielsen, who suggested this work and gave us embedded specimens from *Nephrops* mouthparts; A. Jespersen, J. Lützen, N. Bruce, A. Nørrevang (Kaldbak laboratory, Faroe Islands), B. Neuhaus and A. Hay-Schmidt (both Royal Veterinary and Agricultural University, Copenhagen, Denmark) for valuable help and suggestions; and S. Elle, T. Olesen, E. Schiøtt-Hansen and B. Rubæk for technical support. This work was supported by the University of Copenhagen and the Danish National Research Council.

Sustained and significant negative water pressure in xylem

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DESPITE two centuries of research, the mechanism of water transport in plants is still debated^{1–8}. The prevailing cohesion–tension theory^{2,3}, which states that water is pulled upwards by capillarity in cell-wall pores, remains vulnerable to challenge because its corollary is difficult to prove: that large negative pressures exist in xylem conduits^{4–7}. Recent xylem pressure-probe and z-tube experiments suggest that cavitation limits xylem pressures to above -0.5 MPa, despite the much more negative pressures predicted by the cohesion–tension theory and measured with the standard pressure-chamber method^{4,5,9,10}. Here we show, using centrifugal force to induce negative pressure between -0.5 and -3.5 MPa in intact stems, that xylem conduits remained water-filled and conductive to species-specific pressures ranging from -1.2 to below -3.5 MPa. Results were consistent when stems were air-dried or injected with air. Agreement among these techniques demonstrates that xylem can support large negative pressures, that the pressure chamber reliably measures these pressures, and that cavitation is nucleated by air entry through conduit wall pores.

Negative xylem pressures (Ψ_{px}) can exist only if cavitation, the nucleation of the phase change to vapour, does not occur. Homogeneous cavitation in water theoretically occurs between -80 and -200 MPa^{11–13} and is relatively insensitive to solutes at the concentrations found in xylem sap³. The stability of xylem water above this pressure limit depends on the absence of heterogeneous nucleating sites where water contacts xylem walls or inclusions, and on the exclusion of air by capillary forces in cell-wall pores^{3,14–18}. The xylem pressure-probe^{4,9,10} and z-tube experiments⁵ are ambiguous because they alter the water's environment from its native state, in the former case by the insertion of a glass capillary tube through the xylem conduit wall, and in the latter by modelling the xylem conduit as a z-shaped glass capillary tube centred on a centrifuge and rotated to generate negative pressure. The exposure of water to these foreign surfaces could have caused cavitation at a less negative Ψ_{px} than in intact xylem conduits.

We determined the minimum Ψ_{px} causing cavitation in intact xylem conduits by modifying the z-tube method^{19,20} to spin stem segments rather than glass tubes as suggested previously⁵. Although conduits at segment ends were severed, segments were long enough to include entire conduits. The extent of cavitation was determined by comparing the hydraulic conductivity (k_h , xylem flow rate per pressure gradient) of the segment before and after spinning. Cavitated conduits, although initially vapour-filled, quickly fill with air as gas diffuses from surrounding air spaces²¹; these conduits are then non-conductive even at atmospheric pressure, and so reduce stem k_h ²².

Populus fremontii (Fig. 1a) showed no decrease in k_h after exposure to Ψ_{px} as low as -1.5 MPa. However, all stems were completely cavitated and were non-conductive at or below -1.6 MPa. When *Populus* stems were spun at speeds above the cavitation threshold (-1.0 MPa) for up to 3 h, there was little or no decrease in k_h (Fig. 2). Conversely, when spun below the threshold (-1.8 MPa), stems showed 100% decrease in k_h after as little as 60 s (Fig. 2). Four other species showed different cavitation thresholds of -1.4 MPa (*Salix gooddingii*; Fig. 1b), -1.9 MPa (*Acer negundo*; Fig. 1c), -3.1 MPa (*Abies lasiocarpa*; Fig. 1d), and below -3.5 MPa (*Juniperus monosperma*; Fig. 1d).

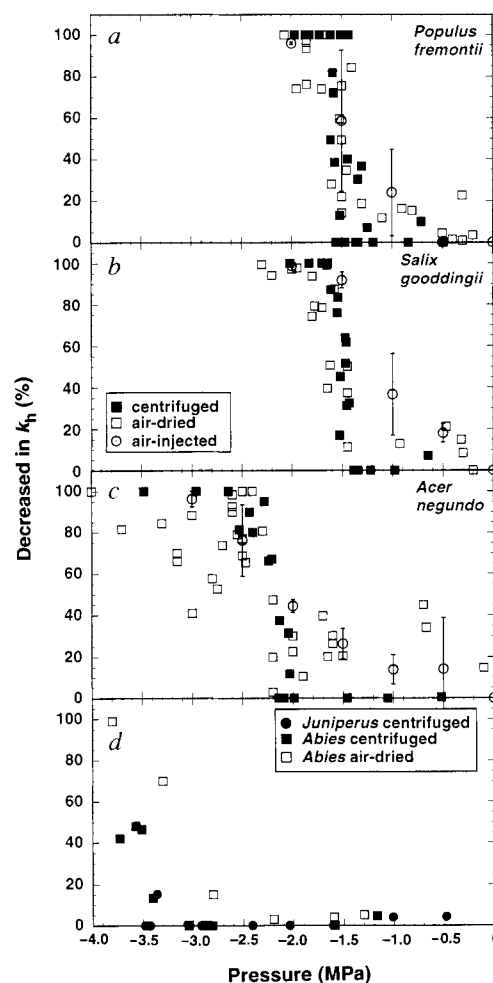


FIG. 1 Decrease in hydraulic conductivity (k_h) in xylem against xylem pressure (Ψ_{px}) generated by centrifuging (filled symbols) and air-drying (open symbols) stems of a, *Populus fremontii*, b, *Salix gooddingii*, c, *Acer negundo* and d, *Abies lasiocarpa*. Centrifuge data only are shown for *Juniperus monosperma* (d). Decrease of k_h from air injection of xylem (a–c, open circles) is shown against the negative of the injection pressure for comparison. All species were from Pima County, Arizona, except *A. lasiocarpa* from the Wasatch Mountains of Utah.

METHODS. In centrifuge experiments, stems were re-cut underwater to a length (26–40 cm) several times longer than all or most of the conduits in each species (W.T.P. and J.S.S., unpublished observations). The bent ends that centre the water column in z-tubes²⁰ were unnecessary because of capillarity at inter-conduit pits. Initial k_h of each segment was measured using a method described previously³⁰. The stem was centred on the rotor of a centrifuge (Sorvall, GLC-1) and spun to generate a pressure (P); $P = 0.5\sigma\omega^2r^2$, where σ is the density of water, ω is the angular velocity (rad s^{-1}) and r is half the total stem length (cm)^{19,20}. Angular velocity was determined using the centrifuge tachometer. After 4 min (2 min for *A. lasiocarpa*) centrifugation, stems were left in damp paper towels for 20–60 min to allow air to diffuse into vapour-filled conduits. Final k_h was measured after removing 2–5 cm from each end under water to eliminate blockage by drained, severed conduits. Cavitation was expressed as the percentage decrease of final k_h from its initial value. The centrifuge was too slow to completely cavitate *Abies* or *Juniperus*. In air-drying experiments, decrease in k_h was calculated from native and flushed k_h ³⁰, and xylem pressure was measured with the pressure chamber. Air-injection experiments were performed as described previously²⁵.

From these data we conclude that negative Ψ_{px} can be sustained for extended periods with little or no cavitation unless Ψ_{px} falls below a defined, species-specific threshold. The higher cavitation pressures seen in pressure-probe^{4,9,10} and z-tube^{5,20} studies appear to be a property of technique rather than of xylem.

We obtained the same relationship between Ψ_{px} and k_h when we air-dried stems and measured Ψ_{px} with the pressure chamber²³ (Fig. 1; compare results for air-drying and centrifugation). The greater scatter in the air-dried as opposed to centrifuged results (especially *A. negundo*) was probably in part a result of pressure-chamber error as stems reached 100% cavitation⁸. However, the general agreement between the methods indicates that the pressure chamber can measure negative Ψ_{px} when cavitation is not extensive.

These results raised the questions of what nucleated cavitation, and why it occurred at different pressures in different species. The 'air-seeding' hypothesis²⁴ states that xylem cavitation is nucleated by air sucked through wall pores when the pressure difference across the air-water meniscus in the pore exceeds that which can be sustained by capillarity. The air-seeding pressure difference will be the same whether the air is pulled in by negative Ψ_{px} as in an intact plant, or pushed in by elevated air pressure. We tested this prediction by measuring the k_h of stems inserted through a steel 'pressure sleeve' as a function of air pressure inside the sleeve²⁵. In support of the hypothesis, the air pressure causing a decrease in k_h in air-injected stems was equal and opposite to the negative Ψ_{px} causing cavitation in air-dried or centrifuged stems (Fig. 1; compare results for air-injected, air-dried, and centrifuged).

Our results join the already considerable evidence supporting the existence of substantial negative pressures in xylem and the validity of the pressure-chamber method for estimating them⁸. By using the same approach of spinning stems, a 1:1 correspondence has been demonstrated²⁶ between centrifugally induced pressure (to -1.6 MPa) and pressure-chamber measurements of leaves attached at the centre of rotation. The pressure chamber was also consistent with Ψ_{px} predicted from pressure-probe measurements of cell turgor (where cavitation is irrelevant) and estimations of osmotic potential of cell sap²⁷. Our evidence for the air-seeding mechanism also corroborates previous studies⁸, and demonstrates that other potential causes of xylem cavitation, such as nucleation at the wall water interface³, are relatively unimportant. Air-seeding will occur through the largest pores in the conduit wall, these being located in the inter-conduit pit membranes²⁴. These thin, porous areas in conduit walls allow inter-conduit passage of water but not a gas-water meniscus; this minimizes air-blockage of xylem when conduits are damaged. The pore diameters in pit membranes range from less than 0.05 to over 0.4 μm (as opposed to <0.01 μm in cell wall proper), depending on species and location in the plant, and their predicted air-seeding pressure has been shown to correspond with

the cavitation pressure in several species^{28,29}. Although the occurrence and mechanism of xylem cavitation is reasonably well documented, its greater significance for determining the drought tolerance, habitat preference and regulation of water use of a species is a matter of continuing study. □

Received 31 July; accepted 29 August 1995.

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ACKNOWLEDGEMENTS. We thank J. Grace, P. Jarvis, J. Passioura, J. Raven and three anonymous reviewers for critiques of the manuscript. This work was supported by a National Science Foundation Doctoral Dissertation Improvement grant to W.T.P. and J.S.S.

Dorsal cell fate specified by chick *Lmx1* during vertebrate limb development

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THE positional cues that govern the fate of cells along the dorsoventral axis of the developing vertebrate limb are established in the mesoderm before outgrowth of limb buds. In *Drosophila*, a LIM/homeodomain gene, *apterous*, expressed in the dorsal compartment of the wing disc, specifies dorsal cell fate^{1,2}. Here we report the isolation of a vertebrate LIM-homeodomain containing gene, Chick *Lmx1* (*C-Lmx1*). Transcripts for *C-Lmx1* are detected in the presumptive dorsal limb mesoderm and are restricted thereafter to the dorsal mesoderm of the developing chick bud. *C-Lmx1* expression is regulated by the overlying ectoderm where *Wnt7a* messenger RNA is localized³. *Wnt7a*, required for normal development of the dorsoventral axis in mouse limbs⁴, can induce ectopic expression of *C-Lmx1* in ventral mesoderm. Misexpression of *C-Lmx1* during limb outgrowth causes ventral to dorsal transformations of limb mesoderm. We propose that *C-Lmx1* specifies dorsal cell fate during chick limb development.

Screening of a chick limb complementary DNA library with a mouse LIM/homeodomain probe produced several LIM/homeodomain clones including *C-Lmx1*. This cDNA encodes a protein that contains two LIM domains linked to a homeobox

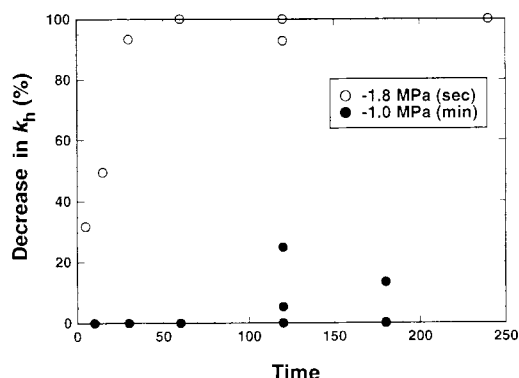


FIG. 2 Decrease in k_h in centrifuged stems of *Populus fremontii* as a function of time at pressures above (filled circles, -1.0 MPa) and below (open circles, -1.8 MPa) the cavitation threshold of -1.2 MPa from Fig. 1a. Note the different time scales (filled circles, minutes; open circles, seconds). Limited cavitation at the longer spinning times above the threshold may have developed from lowering of negative pressure by evaporation during spinning.

motif and shows 70% amino acid identity to the hamster *Lmx1* gene⁵. Transcripts for *C-Lmx1* are first detected around stage 14–15 in the lateral plate mesenchyme of the limb region (data not shown) when limb budding starts, *C-Lmx1* transcripts become localized to the dorsal mesoderm region of both fore- and hindlimbs (Fig. 1a, d) where they are maintained at least until stage 30 (Fig. 1b, c, e, f, and data not shown). *Wnt7a* transcripts start to be detected at approximately the same time as *C-Lmx1* (around stage 14) but in the overlying ectoderm (data not shown), where they are restricted during limb bud outgrowth³ (Fig. 1g–i).

To determine whether *C-Lmx1* is involved in specifying dorsal cell fate during chick limb development, we infected the primordia of limb buds with a replication-competent retroviral vector carrying the *C-Lmx1* gene to ectopically express *C-Lmx*. Ventral to dorsal transformations of cell fate at the more distal parts of *C-Lmx1* infected limbs, affecting the skin, muscle and bones, were observed.

The wild-type chick leg has small scales or tuberculae covering the ventral face of the toes where they form the plantar phalangeal pads (Fig. 2a, b). Large scales or scuta cover the dorsal face of the tarsometatarsus and toes (Fig. 2c, d). The distal tips of the digits have claws with a ventral convexity (Fig. 2b, d). The feather pattern on the dorsal surface of the wing (Fig. 2o) is denser than that on the ventral side (Fig. 2p). All these features are convenient markers to distinguish dorsal from ventral cell types.

After *C-Lmx1* viral infection of stage 7–14 chick embryos ($n = 366$ injected individuals), wings developed supernumerary feathers on the ventral side resulting in a bidorsal feather pattern (28%, $n = 103$; Fig. 2q). When the leg primordium was targeted, the integument of specific toes and/or certain areas of the tarsometatarsus region on the ventral side of the leg showed typical dorsal scales (20%, $n = 72$; Fig. 2i–k) and the tubercular scales did not form characteristic metatarsal and phalangeal pads on

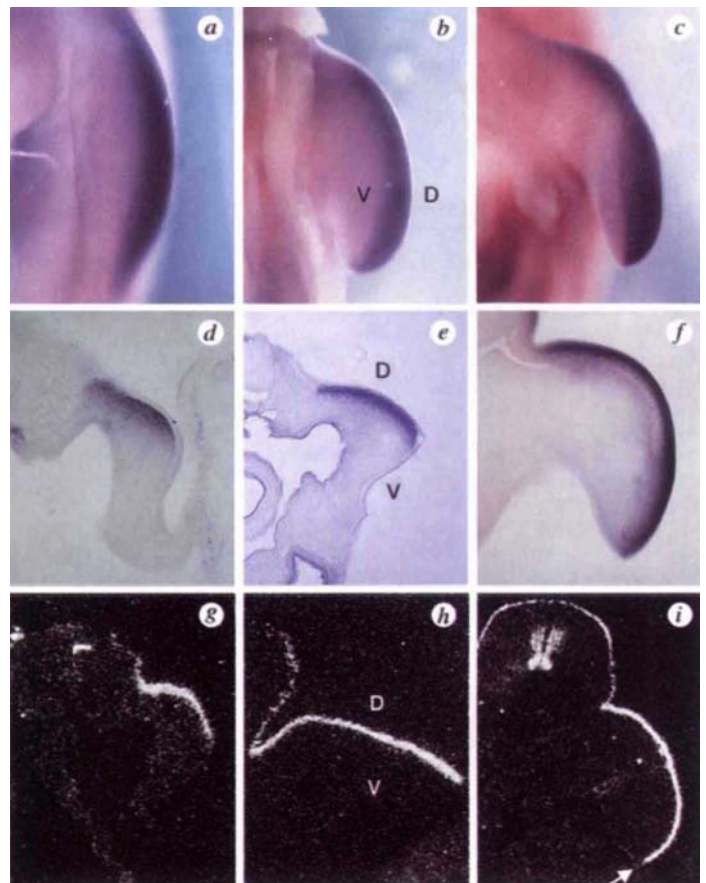
the ventral side of the foot (24%, $n = 89$; Fig. 2h). Some claws did not show the typical ventral curvature, being either straight or dorsally curved (10%, $n = 37$; Fig. 2f, h–k). Abnormalities in dorsoventral polarity of muscles and tendons were evident in the autopod region of infected limbs. In the leg, some ventral muscles of the foot were lost (6 of 10 cases examined). The most affected were the flexor apparatus of digits 1, 3 and 4 (Fig. 3a–d). In some instances, extensor muscles, which characterize the dorsal side and allow the extension of the digit phalanges and dorsiflexion of the foot at the ankle, developed in the ventral side of the digits (4 of 10 cases examined; Fig. 3a, b). Changes were also observed in the wing where the *abductor medius* was often missing (5 of 6 cases examined; Fig. 3e, f). This muscle lies along the anteroventral surface of metacarpal 3 and is involved in the flexion of digit 3. Proper arrangement of muscles and tendons determines the dorsoventral direction of flexure of the limbs. The loss of ventral muscles or the presence of dorsal supernumerary muscles in the ventral side of the autopod could lead to incorrect bending of digits and ankle (Fig. 2e–i, k, m, n), causing the limbs to be straight or dorsiflexed (30%, $n = 110$; that is, in the leg, the top of the foot moves towards the dorsal tibial bone) contrary to their normal ventral curvature.

Digit duplications in wings and legs (Fig. 2g, and data not shown) and development of feathers on the ventral and/or dorsal side of the foot (Fig. 2f, h) were also observed.

To determine whether *C-Lmx1* misexpression phenotypes were related to changes in ectodermal cell fates, we studied, after *C-Lmx1* infection, the expression of *Eng-1* and *Wnt7a*, two markers that during normal development are restricted to the ventral and dorsal ectoderm, respectively^{3,6}. That expression of *Wnt7a* and *Eng-1* was not altered after *C-Lmx1* misexpression (data not shown) suggests that the ventral to dorsal cell fate changes induced by *C-Lmx1* are not attributable to ectodermal changes in cell fate, but rather are restricted to the mesenchyme. Our results confirm the conclusions of previous grafting experi-

FIG. 1 *C-Lmx1* and *Wnt7a* expression on the dorsal side of the developing limb bud. *In situ* hybridization of *C-Lmx1* mRNA in whole mounts (a–c) at stage 18, 23 and 26, respectively, and in sections (d–f) at stages 18, 20 and 25, respectively, of chick limb development²⁰. Expression of *C-Lmx1* is localized to the dorsal limb mesenchyme. g–i, *In situ* hybridization in sections of *Wnt7a* mRNA at stage 18, 21 and 24, respectively, of chick limb development. In all stages analysed, *Wnt7a* transcripts are specifically localized in the dorsal ectodermal layer. D, dorsal; V, ventral.

METHODS. After being fixed in 4% paraformaldehyde overnight, chick embryos were processed for whole-mount and section *in situ* hybridization as described²¹. The DIG antisense probe used covers the entire open reading frame of the chicken *C-Lmx1* clone. Approximately one million phages of a lambda ZAP II cDNA library prepared from stage 20–22 chick limb bud RNA were screened under low-stringency conditions²¹ by hybridization with a mouse LIM/homeo-domain probe. Positive clones were excised and sequenced according to the manufacturer's instructions (Stratagene and USB). Several LIM/homeobox type genes were isolated, including *C-Lmx1*, and these will be published elsewhere. The ³⁵S antisense *Wnt7a* probe used has been described previously³. For histological examination, after whole-mount *in situ* hybridization, embryos were dehydrated in 30% sucrose, embedded in gelatin, frozen and sectioned with a cryostat.



ments which indicate that the control of dorsoventral determination resides in the mesoderm⁷⁻¹⁰.

The limits of expression for both *C-Lmx1* and *Wnt7a* are sharply demarcated by the apical ectodermal ridge (AER), suggesting that signals originating in the AER could account for dorsal restriction of *C-Lmx1* expression. Maintenance of *C-Lmx1* expression is independent of the AER because, 48 hours after AER removal from a growing wing bud (stage 21), *C-Lmx1* transcripts were still confined to dorsal mesenchyme (Fig.

4c). To investigate whether dorsal ectoderm might regulate expression of *C-Lmx1*, the dorsal ectoderm of a stage-22 wing bud was surgically removed from the dorsal mesoderm. Expression of *C-Lmx1* in the underlying mesoderm was barely detected 48 hours later (Fig. 4a, b). This indicates that signals originating in the dorsal limb ectoderm could maintain or activate expression of *C-Lmx1* in dorsal limb mesoderm.

Wnt7a expression is restricted to the overlying ectoderm³, suggesting that it could be a dorsal ectodermal signal mediating

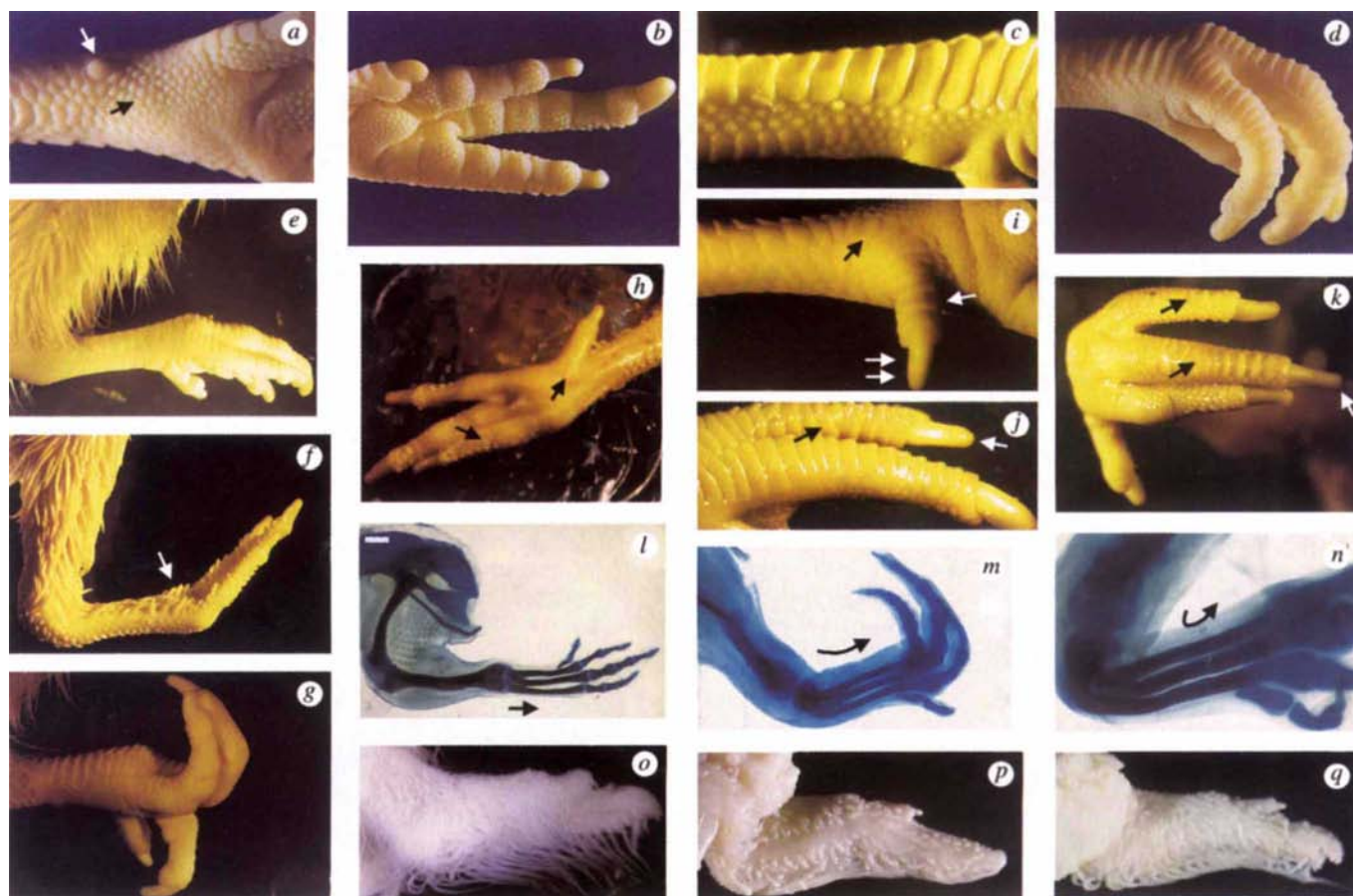


FIG. 2 Ectopic *C-Lmx1* expression causes ventral to dorsal changes of the integument and perturbs bending of the limb. Ventral view of a, the tarsometatarsus and b, the toe region of a 14-day chick embryo illustrating the small scales or tuberculae (black arrow), the typical phalangeal pads, and the ventral curvature of the claws. Dorsal view of c, the tarsometatarsus and d, the toe region of a 14-day chick embryo, showing the large scales or scuta, typical of the dorsal side, and the ventral curvature of the claws. e, Whole-mount of a wild-type chick leg (day 14). f, After *C-Lmx1* injection at stage 12, the claws are straight and the digits are dorsiflexed (white arrow). Feathers, some duplicated, developed on the dorsal and ventral side of the foot. g, Dorsiflexion of digits 3 and 4 following viral injection of *C-Lmx1* into stage-10 chick embryo. The dorsoventral pattern of the skin is unchanged. h, Ventral view of an infected leg where the phalangeal pads have disappeared. The digits are dorsiflexed and ectopic featherbuds developed. i, Ventral view of a *C-Lmx1* infected leg. The tarsal tuberculae of the ventral side are transformed into larger scales typical of the dorsal region (black arrow). Note the absence of the characteristic ventral pad (white arrow in a). Digit 1 shows ventral to dorsal transformation denoted by both the claw orientation (double white arrow) and the absence of phalangeal pads on the ventral side which are replaced by dorsal-type scales (white arrow). j, Following viral *C-Lmx1* injection an extra digit developed with a bidorsal phenotype at the distal tip. The claw is straight (white arrow). k, Ventral view of the phalanges of the foot following viral *C-Lmx1* infection. The most posterior digits, 3 and 4, show bidorsality. Both ventral

and dorsal sides are covered with large scales characteristic of the dorsal side (black arrows). The integument of the ventral region of digit 2 and digit 1 is unchanged although the digits are dorsiflexed. l, Skeletal pattern of normal chick leg (day 10). Note that the bones of the tibia and metatarsals are straight. In the infected legs, the degree of flexion of the foot towards the dorsal tibial bone varies in intensity. m, In mild cases, only the distal part of the phalanges are affected. n, In more severe cases, the bending affects not only the distal phalanges but also the tarsometatarsus joints. o, Dorsal and p, ventral view of a wing from a 12-day, wild-type chick embryo. The dorsal region has a more dense feather pattern than the ventral region. q, Ventral side of a 12-day chick wing after *C-Lmx1* infection. Note the presence of supernumerary feathers throughout the ventral side.

METHODS. Chicken embryos were obtained either from MacIntyre Poultry (San Diego, CA) or from SPAFAS (Norwich, CT). *C-Lmx1* and *Wnt7a* virus production was done as reported previously¹⁵. *C-Lmx1* concentrated viruses were microinjected into the limb primordia areas of stage 10–14 chick embryos. For easier observation of the viral injection into the early chick, fast green (0.2%) was added to the virus stock before injection (1/1,000 dilution). After virus injection, the embryos were left to develop for 7–12 days, and evaluated for evidence of alterations in the integument. For visualizing the cartilage structure, after viral injection, embryos were fixed in 5% trichloroacetic acid overnight and stained with 0.1% alcian green, dehydrated and cleared with methylsalicylate.

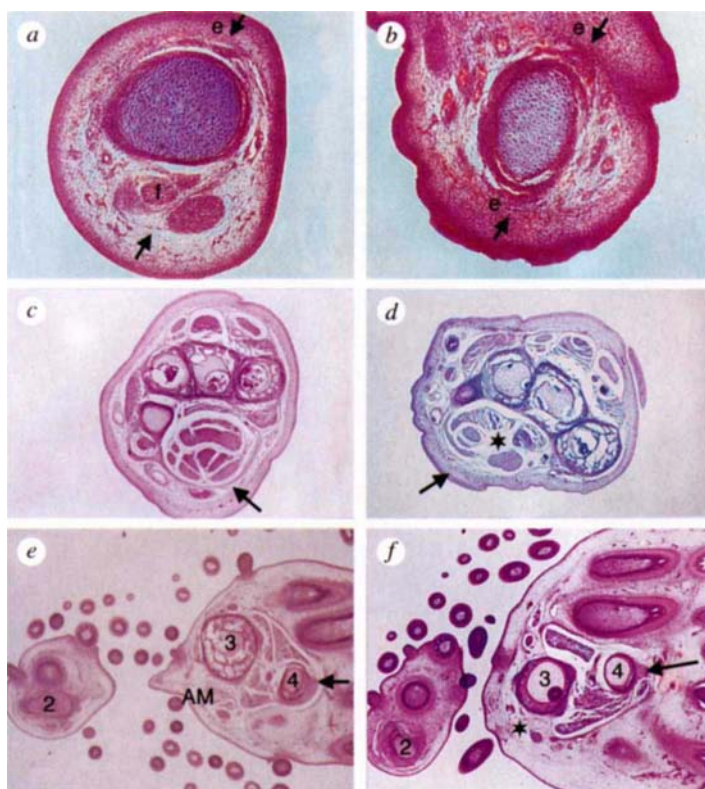


FIG. 3 Ectopic *C-Lmx1* expression causes reorganization of limb muscles. Cross-section of **a**, a normal and **b**, a *C-Lmx1* infected leg digit 1, showing the (f and black arrow) ventral muscle apparatus (*flexor perforatus*, *flexor superficialis* or *perforans et perforatus* and *flexor profundus*) and the (e and black arrow) dorsal muscle, *extensor hallucis longus*. The ventral muscle apparatus is totally absent in the *C-Lmx1* infected leg and replaced by an ectopic (e) dorsal muscle, *extensor hallucis longus*. Note that the bone has a cylindrical shape compared to the wild type. Cross-section of **c**, a normal and **d**, a *C-Lmx1* infected leg just above the distal malleoli of the tarsometatarsus. Although the dorsal muscles are normal in the infected leg, some of the ventral (bottom) flexor muscles (*flexor profundus* and *flexor superficialis*) are missing (asterisk). Note that the position of digit 1 is slightly more dorsal than in the control leg. Cross-section through the mid-autopodium of **e**, a normal and **f**, an experimental wing at the levels of digits 2, 3 and 4 showing the absence (asterisk) of the ventral muscle, *abductor medius* (AM) in the infected wing. Note the duplication of the protuberance of the third metacarpal which may indicate a bidorsal cartilage pattern. Normally, at this level, the fourth metacarpal is slightly ventral relative to the third metacarpal (black arrow). In the infected wing, there is a change in orientation, causing it to be dorsal to the third metacarpal (black arrow).

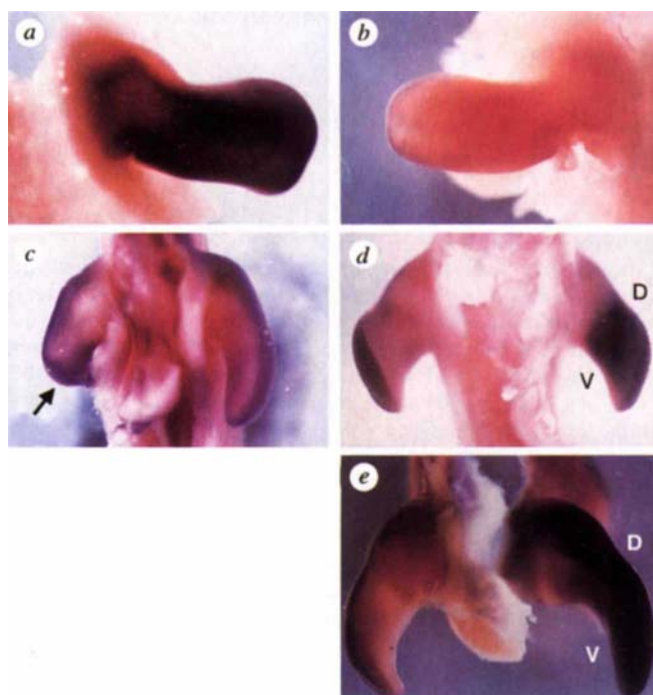
METHODS. After virus injection, embryos were left to develop for 7–12 days and fixed overnight in Bouin's, and dehydrated in 70%, 85%, 95% and 100% ethanol and paraffin embedded. They were serially sectioned at 10 μ m in a microtome, stained with haematoxylin/eosin, and mounted with permount to visualize the muscle pattern.

C-Lmx1 activation and/or maintenance. This was studied by misexpressing *Wnt7a* throughout the limb bud using a replication-competent retroviral vector. *C-Lmx1* expression, normally restricted to the dorsal mesenchyme, was ectopically induced in the ventral mesenchyme 30 hours after *Wnt7a* viral injection (Fig. 4d). This could explain why, after stage 15, limb mesoderm recombined with dorsoventrally reversed ectoderm develops into limbs with the polarity of the epidermal structures reversed^{11,13}. Reversal of the interaction between *Wnt7a* and *C-Lmx1* might cause the disappearance of *C-Lmx1* from the original dorsal

mesenchyme and, reciprocally, induction of *C-Lmx1* in the original ventral mesenchyme. This shift in *C-Lmx1* expression would consequently be responsible for the change in the dorsoventral character of the recombinant limb. This interpretation is consistent with the fact that, in the absence of dorsoventral differentials in the limb mesoderm, as in dissociated and reaggregated limb mesoderm packed into limb ectoderm, the dorsoventral polarity of the epidermal structures conforms with the ectoderm¹³. Dissociation of the mesoderm would abolish the asymmetric expression of *C-Lmx1*, which could be restored by the presence of

FIG. 4 Regulation of *C-Lmx1* expression. **a**, Dorsal view of *C-Lmx1* expression in dorsal limb mesenchyme of an unmanipulated chick limb at stage 26. **b**, Dorsal view of contralateral wing, 24 h after dorsal ectoderm removal at stage 22. Expression of *C-Lmx1* is downregulated in dorsal mesenchyme. **c**, Expression of *C-Lmx1* is unaffected 48 h after AER removal (left wing) at stage 21. **d**, *C-Lmx1*, normally restricted to the dorsal side (D) of the limb bud (left wing), is induced in the ventral mesenchyme (V) by ectopic misexpression of *Wnt7a* (right wing). **e**, Ectopic expression of *C-Lmx1* in the limb. *C-Lmx1* virus was injected into the limb primordia at stage 12, collected and processed for whole-mount *in situ* hybridization with the *C-Lmx1* probe 2 days later. Consistent *C-Lmx1* expression is observed throughout the distal part of the injected limb bud both ventrally and dorsally (note the intensity of the dorsal staining compared with the non-injected contralateral bud). In an average injection experiment, 65–75% of the injected embryos showed a similar level of infection. Ectopic *C-Lmx1* expression later extended to the flank and adjacent somites. Control virus injection showed no morphological alterations.

METHODS. AER (stage 20–21) and dorsal ectoderm removal (stage 22) were as previously reported^{22,23}. Particular care was taken to ensure that mesodermal cells were not removed when the dorsal ectoderm was peeled away. *C-Lmx1* expression was assayed 24 h later by *in situ* hybridization. *Wnt7a* virus (prepared as described previously¹³) was microinjected at stage 17 and processed *in situ* 30 h later for whole mount.



Wnt7a in the apposed ectoderm. In *Drosophila*, *wg*, although expressed ventrally, appears to control the correct dorsoventral compartmentalization of the wing disc by, among other actions, setting the position of *apterous* expression in the dorsal compartment¹⁴. During vertebrate limb development, *Wnt7a* and *C-Lmx1* might interact similarly to specify dorsal cell fate. The *Wnt7a* protein could be released by the dorsal ectodermal cells into the underlying mesenchyme, where it controls *C-Lmx1* expression.

Our results show that misexpression of *C-Lmx1* in ventral mesoderm can change its ventral to dorsal character, suggesting that *C-Lmx1* is sufficient to specify dorsal cell fate during chick limb development. However, that only the most distal parts of the limb appears affected^{4,11,12} (that is, cells not yet specified) in *Wnt7a* mutant mice and in both *C-Lmx1* misexpression and recombinant limbs obtained after ectoderm reversal in chicks suggests the presence of factors other than *Wnt7a* and *C-Lmx1*. Alternatively, it is possible that *C-Lmx1* acts very early in development and, in our experimental set up, misexpression is not totally achieved until after some developmental decisions have been irreversibly completed in the proximal elements of the limbs¹⁵.

Because AER grafts induce outgrowth of double-dorsal or double-ventral character depending on which side of the limb ectoderm is implanted¹⁶, it follows that a single signal cannot pattern the entire dorsoventral axis. Perhaps a localized dorsal gene, possibly *C-Lmx1*, specifies dorsal patterning, and a localized ventral signal, not yet identified, specifies ventral patterning. In mice lacking *Wnt7a* activity, dorsal structures are absent, and double-ventral structures are produced. This could be explained by assuming that a ventral signal is also present in the dorsal side but is normally masked by the presence of a dorsal signal^{4,17}. This model, in which 'ventral' would be the default state, would be analogous to a model proposed to act along the anteropos-

terior limb axis in which the function of posterior *Hox* genes tends to prevail over more anterior genes ('posterior prevalence' or 'phenotypic suppression')^{18,19}. In this view, the 'dorsal prevalence' of *C-Lmx1* misexpression would not only eliminate ventral features but also induce double-dorsal structures. □

Received 30 October; accepted 15 November 1995.

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SUPPLEMENTARY INFORMATION. Requests should be addressed to Mary Sheehan at the London editorial office of *Nature*.

ACKNOWLEDGEMENTS. A.V. and C.R. contributed equally to this work. We thank J. Botas for support and discussions; B. Blumberg for comments; J. Botas for the LIM/homeodomain *apterous* probe; A. MacMahon and L. Niswander for the mouse *Wnt7a* cDNA and cDNA virus; C. Tabin for the chick *Wnt7a* *in situ* probe; A. Joyner for *Eng-1*; S. Alvarez for the *Eng-1* antibody; J. De La Peña for technical assistance; and A. Tugores, V. Hoepker, M. Cohn and I. Moore for support throughout this research. W.W. is a summer volunteer student. This work was entirely supported by The Mathers Foundation.

Chromosome engineering in mice

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CHROMOSOMAL rearrangements are the major cause of inherited human disease and fetal loss¹. Translocations² and loss of heterozygosity³ are important genetic changes causally involved in neoplasia. Chromosomal variants, such as deficiencies, are commonly exploited in genetic screens in organisms such as *Drosophila* because a small portion of the genome is functionally hemizygous⁴. In the mouse, deficiencies are not generally available, thus genetic screens for recessive mutations are cumbersome⁵. We report here that defined deficiencies, inversions and duplications extending to 3–4 cM can be constructed in embryonic stem cells. This was achieved by consecutive targeting of *loxP* recombination substrates to the end points of a genetic interval followed by Cre-induced recombination. This reconstructs a positive selectable marker which facilitates direct selection of clones with a chromosome structure specific to the relative orientation of the *loxP* sites. Duplication and deletion alleles have been transmitted into the

mouse germ line. The availability of mice with defined regions of segmental haploidy will allow their use in genetic screens and enable accurate models of human 'chromosomal' diseases to be generated.

Deficiencies are valuable for detailed analysis of a genetic interval⁶. In the mouse very few deletions are available, and those that are were generated randomly using mutagenesis approaches such as ionizing irradiation⁷. Small deletions (20 kb) have been generated in embryonic stem (ES) cells by conventional gene targeting⁸, but the construction of larger deletions, inversions or duplications has not been possible. Site-specific recombinases have been used to mediate recombination over large distances in *Drosophila*^{9,10}, plants^{11,12} and mammalian cells¹³.

Cre, a recombinase that catalyses recombination between *loxP* sites^{14,17}, was used to engineer chromosomal changes in ES cells. *loxP* sites were targeted to the endpoints of the region that was to be rearranged. Because the absolute frequency with which these deletions could be generated was unknown, the *loxP* sites were embedded in two complementary but non-functional fragments of a hypoxanthine phosphoribosyl transferase (*Hprt*) mini-gene cassette¹⁸. Recombination between the *loxP* sites in *hprt*-deficient AB2.2 ES cells restores the activity of this cassette permitting the direct selection in HAT media of only those cells with the desired chromosomal structure (Fig. 1a, b). The *loxP*-*Hprt* minigene fragments are linked to different positive selection cassettes required for selection during gene targeting (Fig. 1b). The positive selectable markers are positioned so that following recombination they are lost from the deleted chromosome but retained in an inversion or duplication allele.

The deletion strategy was tested at the *HoxB* locus because the gene orientation was known¹⁹. The strategy outlined in Fig. 1c was followed. The *hprtΔ3'* cassette was targeted to *Hoxb-1*¹⁹

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(Fig. 1d). An ES clone with the *Hoxb-1* targeted allele was identified and the *Hoxb-9* locus was targeted with the *hprtΔ5'* cassette (Fig. 1e). The *Hprt*-minigene fragments were configured to lie in the correct order and orientation on the targeted chromosome(s) with the *neo* and *puro* cassettes positioned between the *loxP* sites.

Several independent double-targeted (*Hoxb-1* and *Hoxb-9*) clones were expanded, transiently transfected with a Cre expression cassette and placed under HAT selection. HAT-resistant clones were recovered. Two types of double-targeted clones were distinguishable by the frequency with which Cre induced HAT resistance. One class (type I) yielded HAT-resistant recombinations at frequencies averaging 1×10^{-5} per treated cell, whereas a second class (type II) yielded HAT-resistant clones at a much lower frequency (Table 1b). The HAT-resistant clones derived from type I clones were confirmed to be products of intrachromosomal or sister chromatid recombination (*loxP* sites in *cis*) because the *HoxB* cluster and the *neo* and *puro* cassettes were deleted and segregated (sister chromatid pathway) or lost as an unstable ring (Fig. 1g). HAT-resistant clones derived from type II clones appear to be products of inter-chromosomal or non-sister chromatid recombination (*loxP* sites in *trans*).

The deletion strategy is substantially more complex for larger intervals because the gene order and orientations are not known. The orientation and order of the *Hprt* minigene fragments will determine the type of chromosomal rearrangement that is required to reconstruct a functional *Hprt* cassette. The possible orientations are illustrated in Table 1a, the recombinant chromosomes derived from the different configurations of double-targeted clones include deletions, duplications, inversions, dicentric and acentric chromosomes. These recombinant chro-

mosomes can be distinguished by molecular analysis and/or segregation of the selection cassettes.

ES cell lines were constructed with 1.0 Mb deletions between the *Gastrin*²⁰ and the *E2DH*²¹ loci, a region that is believed to encompass a tumour-suppressor gene on the basis of the conserved linkage group on human chromosome 17q and loss of heterozygosity (LOH) studies²² (Fig. 2). In the absence of a *priori* knowledge of the gene order and orientation, all four possible targeted arrangements of the *hprt* minigene fragments were made and tested (Table 1a). The *hprtΔ3'* cassette was targeted to the *E2DH* locus with the alternative minigene orientations (A or B). Targeted clones representing both A and B orientations were in turn targeted with the *hprtΔ5'* cassette (A or B orientations) at the *Gastrin* locus. Multiple independent targeted clones were isolated representing the four different minigene configurations to ensure that clones with the *cis* and the *trans* configurations were likely to be represented. Each of these clones was expanded, transfected with a Cre expression cassette and plated under HAT selection.

HAT-resistant clones were recovered from each of the four alternative minigene configurations. Individual clones within a specific orientation group could be placed into one of two classes based on the frequency with which HAT-resistant clones could be recovered (Table 1a). Sib selection analysis identified the AA type I HAT-resistant clones as those that had lost the neomycin and puromycin resistance genes; these clones are the most likely to have the deletion. Molecular analysis confirmed that HAT-resistant derivatives from the AA type I clones had the desired deletion. Knowing that the AA clones gave deletions defines the relative orientation and order of the minigene cassettes in the *Gastrin* and *E2DH* loci. Using this information, predictions were

FIG. 1 a, The *Hprt-loxP* minigene cassette. b, The *hprtΔ5'* and *hprtΔ3'* recombination substrates. c–g, General strategy for Cre-induced targeted genomic rearrangements illustrated at the *HoxB* cluster, only the intrachromosomal pathway is shown.

METHODS. a, The PGK*Hprt* minigene¹⁸ was modified by the insertion of a *loxP* site from pBS64 (*Hind*III–*Eco*RI, Klenow blunt) into the unique *Xba*I site (Klenow blunt) in the *hprt* intron. Insertion of the *loxP* site did not disrupt the cassette's HAT resistance function (not shown). b, The *loxP*–*Hprt* cassette was divided into two overlapping pieces: *hprtΔ3'*, which contains the PGK promoter, the *hprt* exons 1 and 2, the *loxP*-intron, and exons 3–6; and *hprtΔ5'*, which contains the *loxP*-intron, *hprt* exons 3–9, and the SV40 poly(A)¹ signal. *hprtΔ5'* and *hprtΔ3'* have a 2.0 kb overlap including the *loxP* site; independently, *hprtΔ5'* and *hprtΔ3'* do not provide HAT resistance, but they do when co-electroporated (not shown). *hprtΔ5'* and *hprtΔ3'* were ligated to positively selectable cassettes (*hprtΔ3'* to the *polI*neo²⁹ gene and *hprtΔ5'* to a PGK-puromycin resistance gene), in both cases the positive markers replace the deleted part of the *loxP*-*hprtΔ5'* cassette, ensuring that, upon recombination, they are separated from the reconstituted cassette. c–g, The general strategy to make the deletions consists of 3 steps. c, The unmodified *HoxB* cluster. d, Step 1, conventional replacement-type gene targeting used to replace the *Hoxb-1* gene with the *hprtΔ3'*-neo cassette. e, Step 2, ES cells identified as correctly targeted are used as a substrate to insert the *hprtΔ5'*-*puro* cassette into the endogenous *Hoxb-9* gene by conventional replacement-style gene targeting, the *cis* configuration is illustrated here. f, Step 3, transient expression of Cre induces recombination between the *loxP* sites which reconstructs a functional *hprt* minigene, the intra-chromosomal recombination pathway is illustrated. g, Cells with the recombinant (deleted) chromosome are positively selected in HAT medium and a chromosomal ring will also be generated by the intra-chromosomal pathway. This is believed to be unstable and lost during the growth of the colony.

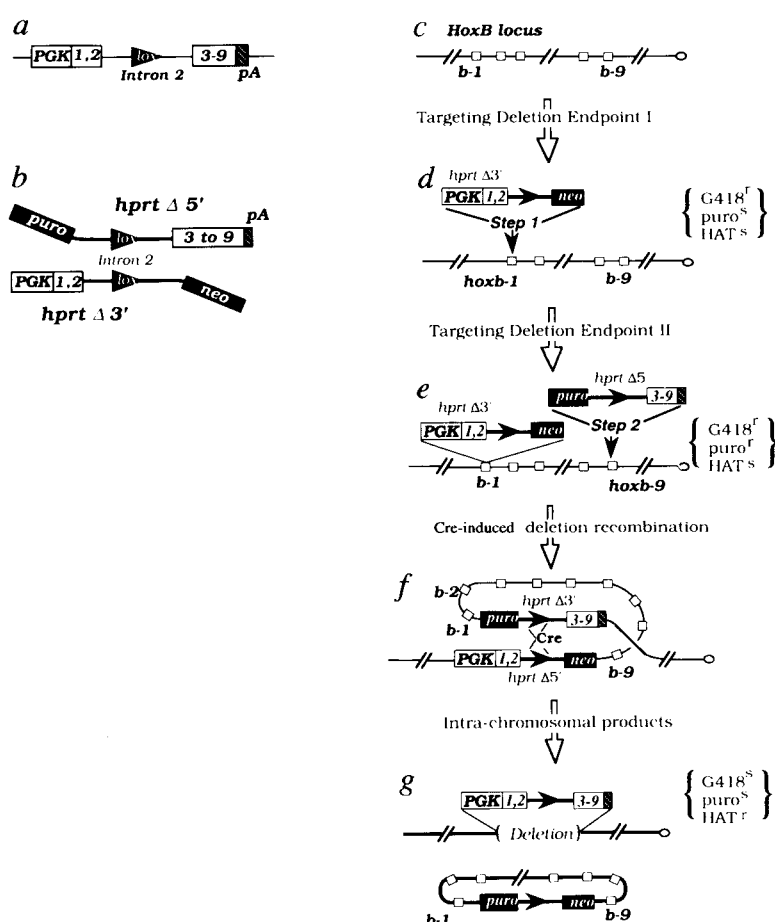
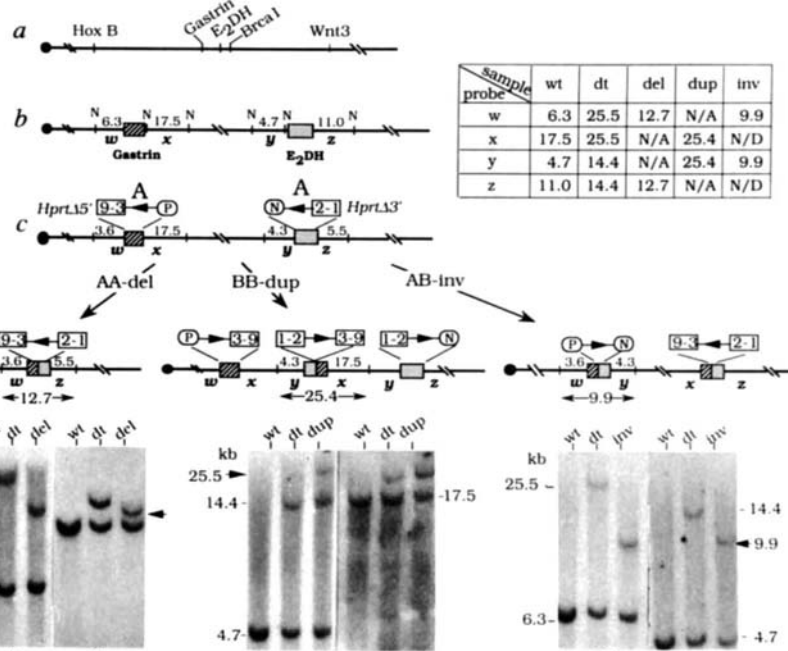


FIG. 2 a, Mouse chromosome 11, the loci used as end points for the chromosome engineering are illustrated. b, *NheI* restriction sites (N) and fragment lengths around the *E₂DH* and *Gastrin* loci. c, Chromosome 11 with both the *E₂DH* and *Gastrin* loci targeted with the *hprtΔ5'* and *hprtΔ3'* vectors, respectively (for clarity just the *cis* configuration and the A orientation is shown). Circled P and N represent the puromycin and neomycin marker genes, respectively. d, The structure of the deletion, duplication and inversion alleles. The sizes of the diagnostic restriction fragments and probes used to detect these alleles are indicated. The deletion, duplication and inversion alleles are derived from the double-targeted chromosome in the AA, BB and AB configurations, respectively. e, Southern blots which confirm the structure of the recombinant chromosomes. The probes used with each blot (w,x,y or z) are indicated beneath each panel and on the diagrams of the various alleles. The lanes are coded as follows wild-type (wt), double-targeted (dt), deletion (del), duplication (dup) and inversion (inv).

METHODS. Overlapping λ phage containing the mouse *E₂DH* locus were isolated from a 129Sv/Ev mouse genomic library using a human *E₂DH* cDNA probe (from B. Ponder). Unlike the duplicated human *E₂DH* locus, the mouse locus is present at a single copy. λ Phage containing the mouse *Gastrin* locus were isolated from the same library using a PCR fragment from the rat *Gastrin* cDNA. The mouse *E₂DH* gene and *Gastrin* genes had not been mapped; to confirm that these genes mapped to mouse chromosome 11, a hamster/mouse hybrid cell line in which the mouse chromosome 11 is the only mouse genetic material (from R.E.K. Fournier) was hybridized to probes specific for the mouse *Gastrin* and *E₂DH* loci. Standard gene replacement targeting vectors were constructed from these genomic clones. *E₂DH* vector: a total of 8.0 kb of homology was used. The *XhoI*-*XbaI* 5.5 kb fragment containing the entire *E₂DH* coding sequence was replaced with the *hprtΔ3'* minigene cassette in both orientations. *Gastrin* vector: a total of 7.5 kb of homology was used. The 3.5 kb *XhoI*-*NheI* fragment containing the *Gastrin* coding region was replaced with the *hprtΔ5'* cassette in both orientations. The two *E₂DH* vectors were separately transfected into AB2.2 *hprt*-deficient ES cells, G418 resistant clones were obtained for each vector. Clones were gridded onto 96-well plates and screened for targeted clones³⁰. Targeted clones were identified at a ratio of 1/25 for the A orientation vector and 1/25 for the B orientation

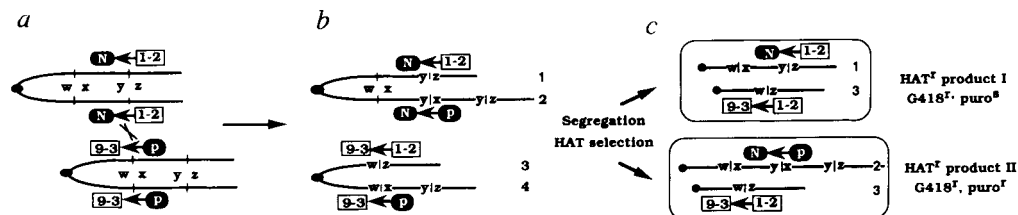


vector. Before subsequent manipulation the two types of targeted ES cell clones were checked by generating chimaeras which were then test bred and scored for germ-line transmission. Totipotent *E₂DH* targeted ES cell lines were identified for both the A and the B orientations and these were transfected with the vectors which target the *Gastrin* locus. Puromycin resistant clones were arrayed on 96-well plates and screened for targeted clones³⁰. All four classes of double-targeted clones were obtained. For simplicity this figure only shows the double-targeted ES cells having the *hprtΔ5'* and *hprtΔ3'* cassettes in *cis*, orientation 'A'. The double-targeted ES cell clones were transfected with the Cre expression plasmid as previously described. HAT^r colonies were recovered and sib selected to test for puromycin and G418 resistance. Individual clones were expanded and analysed for junction fragments using multiple probes. Each blot was hybridized with two probes one from the *E₂DH* locus and the other from the *Gastrin* locus. The junction fragments are indicated with arrows.

made regarding the likely recombinant chromosomes that would be derived from the alternative minigene configurations; BB should generate duplications whereas AB or BA (in which the *loxP* sites are oriented as inverted repeats) should produce inversions (Table 1a). These predictions have all been confirmed by detailed molecular analysis of HAT-resistant clones (Fig. 2).

The AA type II clones yield HAT-resistant recombinants at a low frequency. We hypothesized that in these clones the deletion selection cassettes were targeted in *trans*. Inter-chromosomal recombination would result in both the *neo* and *puro* resistance genes being located on one chromosome whereas the reconstructed *Hprt* minigene would be on the homologue. Thus it

FIG. 3 *G₂* *trans* recombination between homologous chromosomes. a, Individual sister chromatids from chromosome homologues are illustrated still joined at the centromere. One chromosome is illustrated with the *hprtΔ5'* neomycin resistance gene (N) used for targeting. The other homologue was targeted with the *hprtΔ3'* cassette linked to the puromycin resistance gene (P). Cre-induced recombination between *loxP* sites on non-sister chromatids is illustrated by a cross. Reference markers which flank the targeting vector insertion sites are labelled w and x for the *hprtΔ5'* vector and y and z for the *hprtΔ3'* vector. b, The recombinant structure of the sister chromatids. The individual chromatids are numbered 1-4. Chromatid 3 carries the reconstructed *Hprt* minigene and the deletion. c, HAT selection selects for chromatid 3



which will segregate with either chromatid 1 or 2 which carries just the *Neo* or both the *Neo* and *Puro* cassettes, respectively. The chromatid 2+3 segregant carries a duplication and deletion (genetically balanced) and is indistinguishable from the *G₁* inter-chromosomal product. The chromatid 1+3 product carries the deletion and the original single-targeted chromosome, this can only have arisen through the *G₂* pathway.

TABLE 1 Frequency of Cre-mediated recombination

(a) Configuration of recombination substrates and induced chromosome structures

Orientation		Class	Clones tested	m HAT ^r (10 ⁻⁷)	HAT ^r chromosome alteration
<i>hprt</i> Δ5'	<i>hprt</i> Δ3'				
←A—	←A—	I	4	470	del
←A—	←A—	II	2	1	del + dup
←A—	←A—	I	5	344	inv
←A—	→B→	II	1	0	dicen + acen
←A—	→B→	I	3	377	inv
→B→	←A—	II	2	0	dicen + acen
→B→	←A—	I	3	166	dup
→B→	→B→	II	6	1.8	dup + del

(b) Function of distance between the *loxP* sites

Interval	Distance	Frequency (10 ⁻⁷)		
		Deletion		Inversion
<i>Hoxb9-Hoxb1</i>	90 kb	153	0.5	ND
<i>Hoxb9-E₂DH</i>	3–4 cM	6	1	43
<i>Gastrin-E₂DH</i>	1 Mb	470	1.8	334
<i>E₂DH-Wnt3</i>	3–4 cM	30	4	19

a, All of the data is derived from the *E₂DH-Gastrin* double-targeted clones. Alternative orientations (A or B) of the recombination substrates are illustrated in the first column, arrows represent the transcriptional direction of the *hprt*Δ5' and *hprt*Δ3' cassettes. AA corresponds to the minigene cassettes in the right order and same orientation, whereas BB corresponds to the minigene cassettes in the same orientation but in the incorrect order. The centromere is positioned on the left and telomere to the right (column 1). The expected products are described in the last column; del (deletion); dup, duplication; inv, inversion; dicen, dicentric; acen, acentric. For the class II clones, only the recombinant alleles from a cell in G₁ are described. Class I double-targeted clones give a high frequency of HAT-resistant recombinants, whereas class II clones give a low frequency of HAT-resistant clones. Retrospective analysis has revealed that the class I and class II clones have the targeted genes in *cis* and *trans*, respectively. b, Deletion frequencies are illustrated for both class I and class II clones while inversion frequencies are just illustrated from class I clones.

METHODS. Double-targeted *hprt*-deficient AB2.2 ES cells were expanded and 1.0×10^7 cells were transiently electroporated with 20 μg of supercoiled Cre expression plasmid pOG231 plasmid. HAT selection was initiated 24–48 h later.

would be anticipated that all of the positive selection markers would be retained in such a cell. Sib selection identified two classes of HAT-resistant clones which were represented at roughly equal frequencies. One type only retained the *neo* cassette and a second type retained both the *neo* and the *puro* resistance cassettes. The segregation of the *puro* resistance gene from the *neo* cassette is readily explained if Cre induced recombination between non-sister chromatids (Fig. 3). This was confirmed by molecular analysis (data not shown). Although the clones with the duplicated and deleted chromosomes can be generated by either inter-chromosomal or non-sister chromatid exchange, the clones that just carry the *neo* cassette can only have arisen by the non-sister chromatid recombination pathway. These clones have been confirmed to carry both the deletion chromosome and a non-recombinant chromosome with just the *E₂DH* targeted locus (Fig. 3).

The clones with the deletion on one chromosome and the duplication on the other are genetically balanced and therefore the best candidates for germline transmission. Four independent clones were injected into blastocysts, and alleles from three of these clones were transmitted into the germ line, despite three cycles of subcloning and expansion. Mice that are hemizygous for this deletion (1 copy), heterozygous for the duplication (3

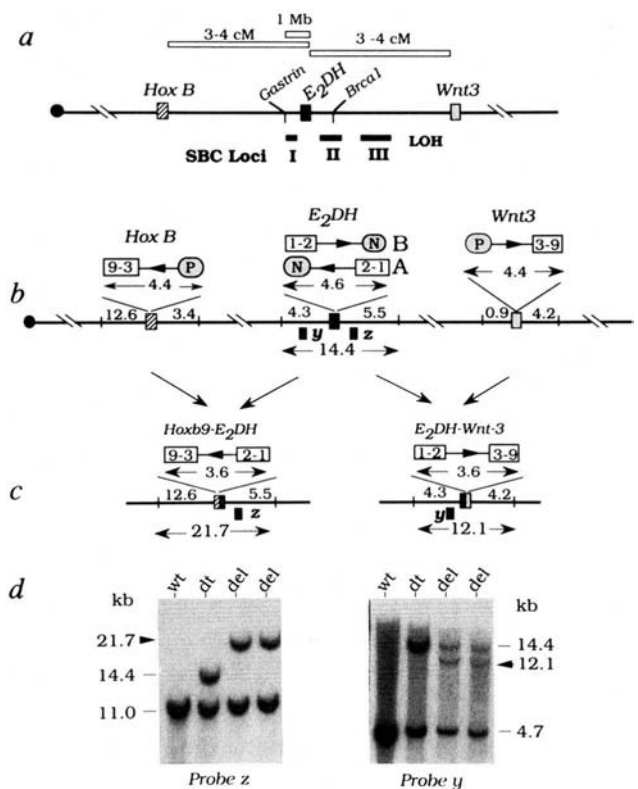


FIG. 4 Deletion of two 3–4 cM intervals on mouse chromosome 11. a, Mouse chromosome 11. The shaded bars indicate the intervals which are to be deleted. *HoxB*, *E₂DH* and *Wnt3* are the loci which serve as the deletion end points. SBC (sporadic breast cancer) loci are indicated by the black bars, these loci are the putative location of tumour suppressor genes based on the analysis of loss of heterozygosity in breast cancer. b, Double-targeted chromosome which has been targeted with the *hprt*Δ5' and *hprt*Δ3' cassettes to the *Hoxb9* and *E₂DH* genes or to the *E₂DH* and *Wnt3* genes. The A orientation *E₂DH* targeted clones were used with the *HoxB-E₂DH* deletion and the B orientation clones were used with the *E₂DH-Wnt3* deletion. Only the orientations of the *hprt*Δ5' cassette which give the deletion products are illustrated. The vertical bars represent *NheI* sites, the sizes of the fragments are indicated and the probes are indicated by shaded boxes labelled x and y. c, The structure of the deletion alleles. Diagnostic *NheI* fragments for the deletion are indicated. d, Southern analysis which confirms the structure of the alleles in the wild-type (wt), double-targeted (dt) and deletion (del) clones. The deletion-specific junction fragments are indicated by the arrows.

METHODS. *E₂DH* and *Hoxb9* vectors have been described previously. *Wnt3* genomic clones were isolated from a 129Sv/Ev genomic library using a cDNA probe²⁸ (gift from Roel Nusse). Conventional replacement targeting vectors containing 7.0 kb of homology was constructed. The *hprt*Δ5' cassette replaces a 2.1 kb fragment (contains exons 3 and 4) of the *Wnt3* gene. The *Hoxb9* vectors and the *Wnt3* vectors were independently targeted into the *E₂DH* targeted cell lines. An 'A' orientation clone was used for the *Hoxb9* targeting vectors and a 'B' orientation clone was used for the *Wnt3* targeting vectors. Transfected cells were selected in puromycin and targeted clones were identified as previously described. Multiple independent double-targeted clones were transfected with the Cre recombinase as previously described. HAT selection was used to isolate recombinant clones which were sib selected and tested by Southern analysis for the predicted junction fragments.

copies) or homozygous for the duplication (4 copies) are all fully viable.

Experiments were performed to investigate if Cre could delete larger fragments. Two 3–4 cM intervals were chosen distal and proximal to the *E₂DH* locus. This region is syntenic with a region on human chromosome 17q that is likely to contain tumour suppressor genes mutated in sporadic breast cancer^{23, 27} in addition to the familial breast cancer gene *Brca1*²³. We have termed

these regions sporadic breast cancer loci (SBCI, SBCII and SBCIII (Fig. 4a). One A or B orientation *E₂DH* targeted clone was selected for the proximal or distal deletion respectively. Targeting vectors with the complementary *hprt-loxP* cassettes were constructed in both orientations for *Hoxb-9* and *Wnt3*²⁸ for the proximal and distal deletions, respectively. Using these vectors double-targeted clones were generated. Cre-induced HAT-resistant clones were recovered (Table 1b). For one of the *Hoxb-9* and *Wnt3* vector orientations, G418- and puromycin-sensitive clones were recovered which were confirmed to have the predicted 3.4 cM deletion (Fig. 4). The vectors with the opposite orientation also yielded HAT-resistant clones which all retained the *neo* and *puro* cassettes. This latter category of clones were all confirmed to be inversions of the 3.4 cM intervals (data not shown).

Deletions ranging from 90 kb to 3.4 cM have been generated on mouse chromosome 11. In total about 10% of chromosome 11 has been deleted using the methods described here. Deletions have great utility as a genetic tool because the diploid genome can be reduced to areas of segmental haploidy allowing genetic screens for recessive mutations. If a panel of deletions was available for a substantial portion of the mouse genome, genetic screens could be contemplated either by scoring phenotypes in mice or by examining mutated cells for specific phenotypes in culture. □

Received 22 September; accepted 23 October 1995.

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ACKNOWLEDGEMENTS. R.R.S. and P.L. contributed equally to this work; the order of citation is arbitrary. We thank S. Sharon for defining the physical distance of the *Gastrin-E₂DH* interval and E. Regel for technical assistance, H. Bellen, J. Lupski and L. Donehower for comments on this manuscript and S. O'Gorman for the timely gift of the Cre expression plasmid pOG231. This work was supported by grants from the NIH. P.L. was supported by a pre-doctoral fellowship from the Markey Foundation. A.B. is an Associate Investigator with the Howard Hughes Medical Institute.

Essential function of LIF receptor in motor neurons

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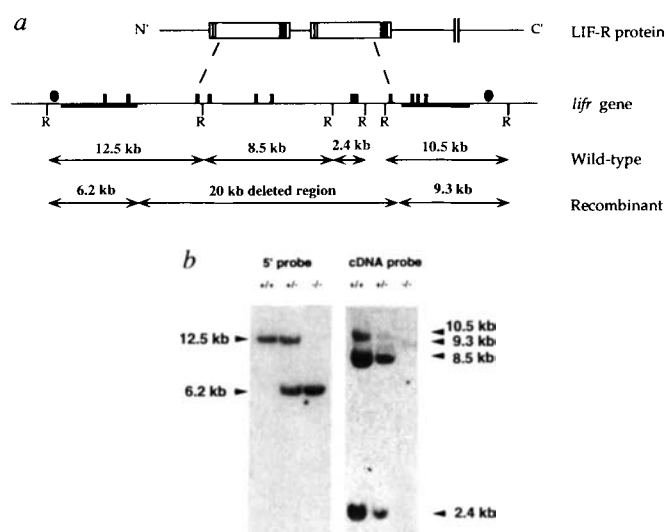
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DEVELOPMENT and maintenance of the mammalian nervous system is dependent upon neurotrophic cytokines. One class of neurotrophic factor acts through receptor complexes involving the low-affinity leukaemia inhibitory factor receptor subunit (LIF-R)^{1–3}. Members of this family of cytokines, such as ciliary neurotrophic factor (CNTF) and leukaemia inhibitory factor (LIF), have profound effects on the survival and maintenance of motor neurons^{4–10}. Recently it was reported that mice lacking LIF-R die shortly after birth¹¹ unlike mice lacking CNTF or LIF which are viable. Here we describe histopathological analyses of *lifr* mutants that reveal a loss >35% of facial motor neurons, 40% of spinal motor neurons and 50% of neurons in the nucleus ambiguus. These findings point to the existence of a ligand for LIF-R that is required for the normal development of motor neurons in both brainstem nuclei and spinal cord.

A deletion of ~20 kb of the *lifr* gene was created by homologous recombination in embryonic stem (ES) cells (Fig. 1). The deleted sequence contains seven exons that encode most of the presumptive ligand-binding region. This was replaced by an internal ribosome entry site-*lacZ/neo* cassette (IRESβ-geopA)^{12,13}, thereby introducing a histochemical reporter into

the locus. The mutant allele was established on hybrid 129/CBA and 129/MF1 backgrounds by appropriate matings of transmitting chimaeras.

Breeding of the mutated allele to homozygosity resulted in the production of liveborn homozygous pups on both genetic backgrounds. The mutant animals were smaller and had a 20% reduction in body weight (1.33 ± 0.22 g, $n=7$) compared with heterozygous (1.67 ± 0.19 g, $n=18$) and wild-type (1.67 ± 0.22 g, $n=8$) littermates at birth. They lacked vigour, did not feed and rapidly became moribund. Although they exhibited limb movements, they were incapable of righting. In all cases so far, homozygotes have died within 24 h of birth and usually within the first 12 h. Similar findings were recently described by Ware *et al.*¹¹.



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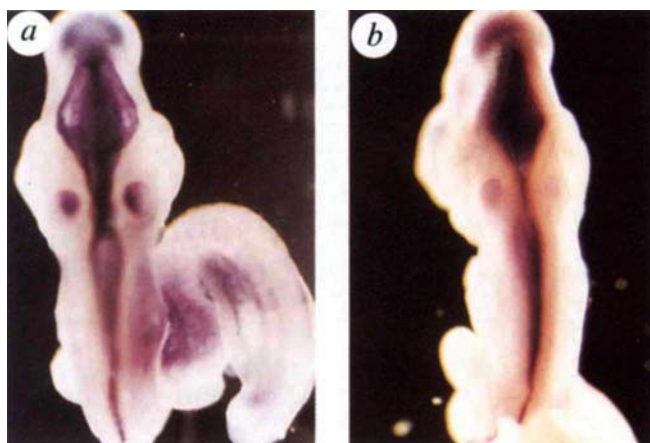


FIG. 2 Expression of integrated β -galactosidase reporter in the 9.5-day mouse embryo and neonatal brain stem and spinal column. *a, b*, Whole-mount *in situ* hybridizations of 9.5-d mouse embryos: *a*, wild-type embryo hybridized with antisense *lifr* probe; *b*, heterozygous embryo hybridized with antisense *lacZ* probe. *c–g*, Localization of sites of Xgal staining derived from expression of β geo integrated into the *lifr* gene. *c, d*, neonatal brainstem regions. Dotted regions mark (*c*) the facial and (*d*) the ambiguous nuclei. Scale bar, 400 μ m. *e*, Neonatal spinal cord and dorsal root ganglia. Scale bar, 100 μ m. *f*, Neurons of the nucleus ambiguus of a *lifr*^{-/-} newborn mouse showing prominent Xgal staining. Scale bar, 50 μ m. *g*, Neonatal spinal motor neurons. Scale bar, 25 μ m. METHODS. *a, b*, Whole-mount *in situ* hybridizations were made by using digoxigenin-labelled probes²⁵. The *lifr* probe was generated by antisense transcription of the 980-bp cDNA fragment described in Fig. 1. The 1125-nt β -galactosidase probe was complementary to sequence downstream of the unique *SacI* site in the *lacZ* gene. *c–g*, Tissue processing and Xgal staining for β -galactosidase on 12- μ m cryostat sections was performed as described²⁶.

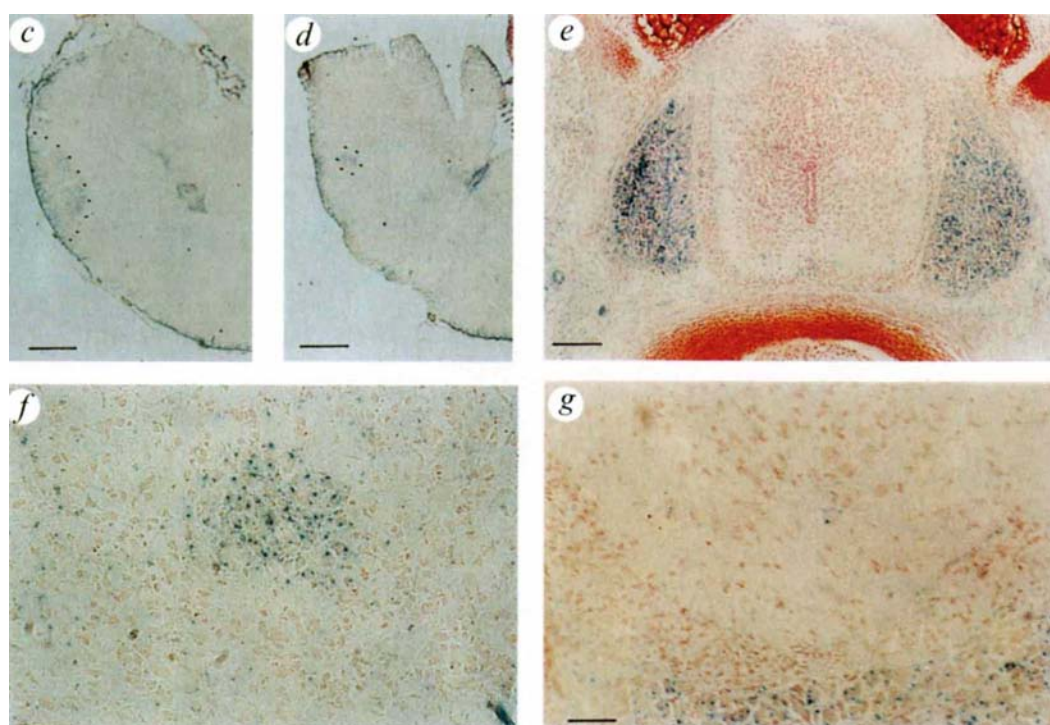


FIG. 1 Targeted distribution of the *lifr* gene. *a*, Deletion strategy. Top, schematic representation of LIF-R protein showing the two cytokine receptor domains boxed. Vertical lines indicate the conserved cysteine residues, and solid bars represent the WSXWS motifs. Bottom, partial genomic structure of the *lifr* gene showing homology arms (thick lines) included in the targeting vector, positions of *EcoRI* restriction sites and sizes of diagnostic restriction fragments. Filled circles indicate the positions of flanking probes used to confirm replacement targeting events. The deletion region contains seven exons encoding amino acid residues 85–444 in the cytokine receptor domains of LIF-R. This region was replaced by an *en-2* splice acceptor-IRES- β geopA cassette¹², flanked by *EcoRI* restriction sites. *b*, Hybridization analysis of tail DNA from wild-type, heterozygous and homozygous *lifr* mutant mice. DNA samples were digested with *EcoRI* and sequentially hybridized with a 5' genomic probe external to the targeting construct (left) and a cDNA fragment (right). The genomic probe was an 800-bp *EcoRI*/*Bam*HI fragment shown in *a*. The cDNA probe was an internal 980-bp fragment that contains a sequence from the deleted exons 3–9 plus a sequence from exon 10. The 9.3-kb band present in heterozygotes and homozygotes

corresponds to the recombinant junction fragment that includes exon 10. METHODS. *a*, *lifr* genomic clones were isolated from a 129Sv genomic library and the positions of exons determined (M.L., A.S., A. Cozens and I. Chambers, unpublished results). A promoterless targeting vector was constructed by subcloning the 5.5-kb *Bam*HI/*Sal*I 5' fragment and the 5.0-kb *Sma*I/*Sac*I 3' fragment into pGEMEM (Promega) and inserting the GTIRES- β geopA cassette. The construct was excised from the plasmid backbone by digestion with *Not*I and *Sfi*I and electroporated into CGR8 ES cells maintained in the absence of feeders²³. After selection in G418 the correct replacement event was identified in 19/58 colonies by filter hybridization with both 5' and 3' flanking probes. Single integration events were confirmed by hybridization with an *en-2* probe. *b*, Two clones injected into C57BL/6 blastocysts gave rise to germline transmission of the mutated allele. Animals were typed by hybridization analysis of tail DNA and/or by β -galactosidase-staining of ear biopsies²⁴. Heterozygous offspring were backcrossed to the appropriate parental strain before intercrossing at the F2 and F3 generations. Homozygous offspring were identified and the deletion was confirmed by DNA filter hybridization.

The differentiation and survival of motor neurons *in vitro*^{4,7,9} and *in vivo*^{5,6,10} have been shown to be strongly enhanced by CNTF and LIF, indicative of a requirement for LIF-R function. The inert condition of LIF-R-deficient neonates was suggestive of possible neuronal dysfunction. Therefore expression of LIF-R within the nervous system and the effects of gene ablation on motor neuron populations were investigated in more detail.

The fidelity of the reporter integrated into the *lifr* gene was established by the close similarity between hybridization patterns obtained *in situ* with antisense LIF-R and β -galactosidase probes and the correspondence observed between presence of IRES- β geo fusion transcripts and histochemically detectable β -galactosidase activity (Fig. 2, and results not shown). Expression in the developing nervous system was apparent in the hindbrain, cranial neural crest and neural tube of 9.5-day embryos (Fig. 2a, b). The distribution of β -galactosidase activity in embryonic (14.4-day), neonatal and adult brain was then examined. Specific staining was evident in cells of the subependymal zone, ependyme and glia limitans. Expression was prominent in neurons in the brainstem motor nuclei, including the hypoglossal and facial nuclei (Fig. 2c, and results not shown), and also in the nucleus ambiguus (Fig. 2d, f). In sections through embryonic and neonatal spinal column, β -galactosidase staining was most pronounced in sensory neurons of the dorsal root ganglia but could also be detected in most of the spinal motor neurons (Fig. 2e, g, and results not shown). In addition some non-neuronal cells were stained, notably astrocytes of the glia limitans in the central nervous system and probably also Schwann cells in peripheral nerves.

The significance of the expression of LIF-R in spinal motor neurons and brainstem motor nuclei was investigated by counting motor neurons in the lumbar (L1–L6) spinal cord and facial nuclei of neonatal homozygous, heterozygous and wild-type littermates. The high expression of the β -galactosidase reporter found in the nucleus ambiguus also prompted an analysis of this structure. Motor neurons within the nucleus ambiguus innervate the oesophagus, pharynx and larynx, and coordinate swallowing. In addition there are neurons in the nucleus ambiguus of rats and mice that generate respiratory rhythm^{14,16}. Data presented in Fig. 3 and Table 1 show that the numbers of motor neurons in LIF-R-deficient animals were reduced by >35% in the facial nucleus and by >40% in the lumbar spinal cord. The nucleus ambiguus neurons were reduced by over 50%. Increased numbers of apparently dying cells with pyknotic nuclei were evident in both spinal-cord and brainstem

TABLE 1 Numbers of spinal motor neurons and neurons in facial and ambiguous nuclei of newborn wild-type, heterozygous and LIF-R-deficient mice

	+/+	+/-	-/-
Lumbar region (L1–L6)	3,213 $\pm$ 230 (n = 3)	N.D.	1,856 $\pm$ 251 (n = 5)
Facial nucleus	3,108 $\pm$ 310 (n = 6)	3,377 $\pm$ 394 (n = 5)	1,951 $\pm$ 135 (n = 11)
Nucleus ambiguus	883 $\pm$ 82 (n = 5)	705 $\pm$ 34 (n = 3)	406 $\pm$ 32 (n = 5)

The lumbar spinal cord and brainstems of newborn mice or mice delivered by caesarian section at day 20 after conception were fixed by transcardial perfusion or immersion fixation with 4% paraformaldehyde. Paraffin serial sections (7 μ m) were prepared and processed as described^{5,6}. After Nissl staining, the number of neurons was counted in every tenth section of the lumbar (L1–L6) region of the spinal cord and every fifth section of the brainstem nuclei. Only neurons with distinguishable nucleus and nucleolus and with clearly identifiable Nissl structure were counted. Average diameters of the nucleoli were as follows. Facial motor neurons: +/+ mice, 2.64 $\pm$ 0.43 μ m; +/- mice, 2.32 $\pm$ 0.22 μ m; -/- mice, 2.14 $\pm$ 0.16 μ m; neurons of the nucleus ambiguus: +/+ mice, 2.36 $\pm$ 0.36 μ m; -/- mice, 2.34 $\pm$ 0.41 μ m (means $\pm$ s.d. for at least five determinations). The counts of facial and ambiguous neuronal nucleoli were corrected for double counting of split nucleoli. Spinal motor-neuron counts are uncorrected. N.D. not determined. Values shown are means $\pm$ s.e.m. of each group. Numbers of neurons are different between +/+ and -/- mice for each group ($P < 0.005$, Student's *t*-test).

nuclei in paraffin sections from homozygotes (Fig. 3d, and results not shown). This suggests that elevated cell death of motor neurons is taking place around the time of birth.

Loss of facial motor neurons should not affect the viability of newborn mice. Impairment of the nucleus ambiguus is of greater significance. These neurons coordinate movements of the oesophagus and pharynx and are therefore required for suckling. Integrity of the nucleus ambiguus is also essential for generation of respiratory rhythm¹⁶. It is probable that loss of these neurons is a contributory factor to the early mortality observed in LIF-R-deficient mice. It would be of interest to know whether this structure is affected in other mouse mutants that die shortly after birth and exhibit reduced numbers of neurons in other brainstem nuclei.

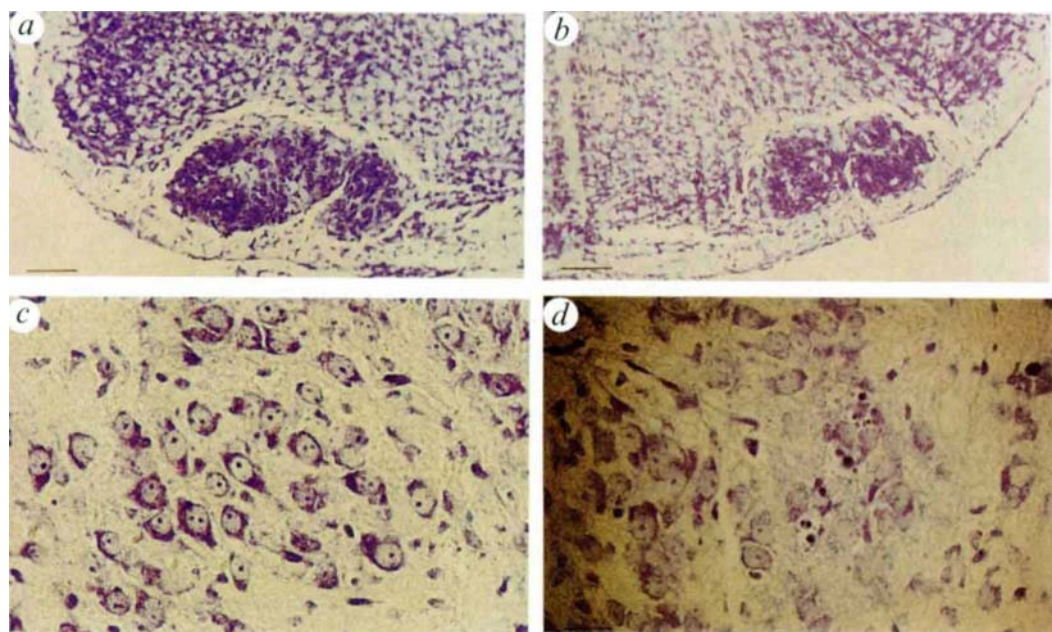


FIG. 3 Neuronal morphology in the facial nucleus of newborn wild-type and *lifr* mutant mice. Facial nucleus of wild-type (a, c) and homozygous *lifr* mutant (b, d) newborn mice from the same litter. Scale bars: a, b, 100 μ m; c, d, 25 μ m.

METHODS. Paraffin sections (7 μ m) were prepared from brainstems of control and *lifr*^{-/-} mice and Nissl stained^{5,6}.

The motor-neuron losses and deficits in the nucleus ambiguus of LIF-R-deficient mice establish that these neurons are dependent on signalling through this receptor during embryonic development. Mice lacking CNTF do not show loss of motor neurons before adulthood⁶ and no severe neuronal defects have been observed in LIF-deficient mice (refs 17, 18 and M.S., unpublished observations). Mice deficient for both CNTF and LIF are viable and do not show functional motor defects at birth (M.S., unpublished observations). The lesions present in newborn LIF-R-deficient animals must therefore reflect the activity of one or more distinct cytokines

active during embryogenesis. Candidates include the recently described cardiotrophin-1 (ref. 19) and a putative mouse orthologue of oncostatin M²⁰. Interaction with the ligand may also involve the CNTF-receptor α (CNTF-R α) subunit, which associates with LIF-R after CNTF binding³. CNTF-R α is expressed in the developing nervous system, in particular in motor neurons²¹, and a preliminary report indicates that its deletion results in developmental defects more severe than produced by deletion of CNTF²². The unidentified cytokine may therefore be a novel ligand for CNTF-R α in addition to LIF-R. □

Received 21 July; accepted 27 October 1995.

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ACKNOWLEDGEMENTS. M.L. and M.S. contributed equally to this work. We thank I. Chambers for LIF-R cDNA, J. Nichols and J. Ure for chimera production, A. Jeske for expert animal husbandry, and H. Döppler for excellent technical assistance; T. Taga for discussion, and I. Chambers and H. Niwa for comments on the manuscript. This research was supported by the Biotechnology and Biological Sciences Research Council, UK, and the Deutsche Forschungsgemeinschaft, Germany.

Suppression of psychoactive effects of cocaine by active immunization

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COCAINE is a powerfully addictive substance and new strategies are needed to treat its abuse. Generating an active immunization^{1,2} to cocaine offers a means of blocking the actions of the drug by preventing it from entering the central nervous system, and should have fewer side effects than treatments based on manipulation of central neurotransmitter function. The design and preparation of a cocaine immunogen requires special regard for the stability of cocaine both free and as a haptenic determinant. Immunochemistry and a well defined behavioural model were brought together to address the problem of inactivation of the psychostimulant actions of cocaine. We report here that active immunization with a new, stable cocaine conjugate suppressed locomotor activity and stereotyped behaviour in rats induced by cocaine but not by amphetamine. Moreover, following acute injection of cocaine, levels of cocaine in the striatum and cerebellum of the immunized animals were lower than those of control animals. These results suggest that immunopharmacotherapy may be a promising means by which to explore new treatments for cocaine abuse.

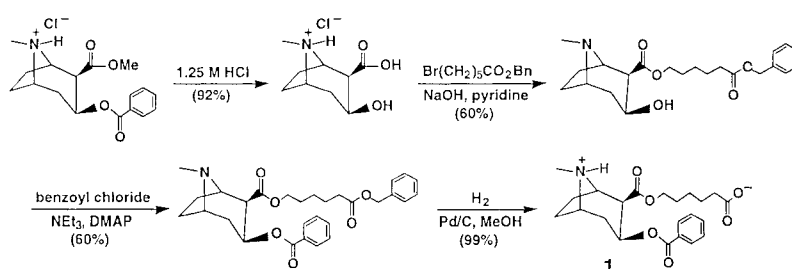
The design and preparation of a cocaine immunogen requires special attention to the stability of free cocaine in solution and as a haptenic determinant. Cocaine degrades spontaneously *in vitro* and *in vivo*^{3,4} largely through hydrolysis of the methyl ester

to produce the non-psychoactive compound benzoylecgonine⁵. Nonspecific esterases are also known to contribute to the *in vivo* degradation of cocaine through indiscriminate cleavage of both the methyl and benzoate esters⁶. Conjugates that have epitopes structurally similar to those of metabolites, especially benzoylecgonine, would compromise the avidity and specificity of a cocaine-specific immune response^{7,8}. Also, an appreciable benzoylecgonine titre would be exceptionally detrimental because the antiserum would be inadequate to neutralize cocaine, particularly in the presence of rapidly formed and stable metabolites^{4,8}. Although each retains the phenyl ring as a major recognition element, the neutrality of cocaine contrasts with the negatively charged benzoylecgonine, a factor in antibody binding, making it possible to maximize the affinity and selectivity for cocaine. By joining the carrier protein to the cocaine framework using a linker at the position occupied by the methyl ester, any minor decomposition of the linked hapten results not in a benzoylecgonine response, but primarily in non-haptenic recognition. Attention to such aspects of the immunochemistry must be emphasized in view of an unsuccessful report of a potential cocaine prophylactic^{9,10}. The hapten GNC (compound 1) was synthesized in four steps starting from (–)-cocaine (Fig. 1). The key reaction, alkylation of (–)-ecgonine, introduced the required tether. The stereochemical configuration remained intact at C2 of the tropane nucleus. This ester linker mimics the alkyl character of the methyl ester of cocaine which is important for recognition of this part of the molecule. Coupling of 1 to keyhole limpet haemocyanin (KLH) afforded the conjugate, GNC-KLH, for immunization.

To assess the efficacy of immunization, the psychostimulant effects of cocaine were measured in the rat. This psychostimulant effect is a dose-dependent increase in locomotor activity and stereotyped behaviour believed to result from cocaine's actions on dopaminergic neurons in the ventral forebrain and striatum¹¹. Male Wistar rats were first tested in photocell cages after treatment with intraperitoneal (i.p.) cocaine-HCl (15 mg kg^{–1}) to determine pre-immunization drug response (baseline). This dose of cocaine is an intermediate dose that produces a significant locomotor activation and modest stereotyped behaviour. Lower

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FIG. 1 Synthesis of the hapten used to form cocaine immunoconjugates. The compound **1** (20 μ mol) was activated for coupling in dimethylformamide (DMF) (200 μ l) using an aqueous solution of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) (26 μ mol) and *N*-hydroxysulphosuccinimide (sulpho-NHS) (26 μ mol) (Pierce). The aqueous content was 10%. After 20 h at 22 °C the solution was added to KLH (20 mg) in 4 ml phosphate buffer (50 mM; PB), pH 7.5. This was kept at 4 °C for 20 h during which time turbidity developed. The fine suspension was dialysed against two changes of 100 mM PB, pH 7.0, in which it could be frozen and stored at least 2 years. Propionic acid was activated and coupled in an identical fashion. This conjugate served as the KLH control for immunization. The same protocol was used to couple **1** to bovine serum albumin (BSA) affording a non-turbid solution of the conjugate used for ELISA. In addition, both KLH and BSA were modified as above with (carbonyl-¹⁴C-benzoyl)-**1** to determine the extent of labelling and stability of the conjugate. The number of ligands were 29 and 19, respectively. Both conjugates were completely stable in 100 mM PB, pH 7.4, 22 °C for at



least 48 h. Finally, competitive binding studies demonstrated that the immune response was highly specific for cocaine. The discrimination was greater than 900-fold versus benzoylcegonine and there was no detectable inhibition of cocaine-binding by ecgonine methyl ester. Consequently, inhibition of cocaine-binding immunoglobulins would not be expected *in vivo* from these metabolites.

doses produce less locomotor activity and virtually no stereotyped behaviour. Higher doses also produce less locomotor activity but more robust stereotyped behaviour¹². Experimental animals were injected 3 days later with GNC-KLH i.p. as an emulsion in RIBI adjuvant and control animals treated with an emulsion containing only KLH. This primary inoculation was followed by boosts at 21 and 35 days. No stimulation of behavioural activity or other behavioural effects were observed after each immunization, consistent with the inability of the conjugate to cross the blood-brain barrier. Serum samples were assayed 7 days after each boost. Titres were typically 1:24,000 by enzyme-linked immunosorbent assay (ELISA) and 1:50 in a solution radioimmunoassay (Fig. 2). Following the final boost of GNC-

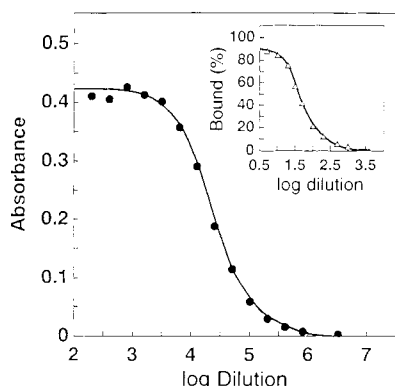


FIG. 2 ELISA and radioimmunoassay (inset) titre measurements of serum from rats immunized with GNC-KLH. ELISA plates (96-well) (costar 3590) containing 2.75 ng per well GNC-BSA in 50 μ l of 10 mM phosphate buffer/150 mM NaCl (PBS), pH 7.4 were dried overnight at 37 °C. This was followed by routine ethanol fixing and blocking with blotto (non-fat milk) in PBS. Rat serum was serially diluted beginning with a 1:200 dilution in blotto. Primary antibody (serum) binding was allowed to take place for 1 h in a moist chamber at 37 °C. After washing, 200 ng per well goat anti-rat IgG conjugated with alkaline phosphatase (Southern Biotechnologies Associates) in 50 μ l PBS-blotto was added and incubated for 1 h in a moist chamber at 37 °C. The plates were thoroughly washed with water, air dried and developed by adding 100 μ l per well of 200 μ M *p*-nitrophenylphosphate (Pierce) in 100 mM 4-morpholinepropanesulphonic acid (MOPS), pH 7.4. After 3 h at room temperature, the absorbance was measured at 405 nm in a microplate reader (Molecular Dynamics). Inset, Radioimmunoassay was conducted in PBS, pH 7.4, in the presence of 5% dimethylsulphoxide (DMSO), 22 °C. [³H]-cocaine (specific activity, 0.234 Ci mmol⁻¹, prepared from norcocaine and [³H]-methyl iodide (Amersham)), to give a final concentration of 0.33 μ M, was added to varying dilutions of rat serum, capable of binding more than and less than 50% of the offered counts. The samples were then treated as in refs 13–16.

KLH or KLH, the animals were challenged with systemic cocaine and their locomotor responses and stereotyped behaviour were measured (first cocaine challenge). The ambulatory response (crossovers) to cocaine was significantly different between control and immunized animals (Fig. 3). This measure was 42% lower in the experimental group compared to baseline (pre-immunization (957.02 $\pm$ 210.42)), whereas the control group displayed a 30% increase compared to baseline (pre-immunization (759.56 $\pm$ 192.37)). A similar decrease in the psychostimulant actions of cocaine was seen in immunized animals upon two subsequent cocaine challenges with differences in locomotion between groups significant at the time of the first and second challenges, but not the last (Fig. 3). Stereotyped behaviour was suppressed in experimental animals consistent with the decreases in locomotor activity and this effect was significant across all challenges (Fig. 3). The antibody titre, as measured 7 days before the final inoculation, was essentially unchanged 8 days after the last challenge.

Analysis of serum immunoglobulin dissociation kinetics, antigen-binding capacity and the effect of antigen dilution^{13–16} were consistent with an *in vivo* antibody excess having a micromolar average binding constant. In addition, comparison with a purified anti-cocaine murine monoclonal antibody indicated that 1 ml undiluted serum from an immunized rat could bind the same amount of cocaine as 4 mg ml⁻¹ monoclonal antibody (M.R.A.C. *et al.*, manuscript in preparation). To complement these experiments, plasma cocaine levels were determined by HPLC (modification of ref. 17) following a 15 mg kg⁻¹ i.p. injection in a separate group of animals. A measured peak concentration of 5.56 $\pm$ 0.31 μ M occurred at 5 min which decayed with a half-life of 25 min. Taken together, the data suggest that at equilibrium at least 50% of the cocaine present in the blood under physiological conditions is likely to be bound.

To support the hypothesis that the observed psychomotor suppression was specific to cocaine, a second group of animals was immunized as described above, then challenged with amphetamine (0.75 mg kg⁻¹) i.p. 3 days after the last boost. Amphetamine stimulates locomotor activity by facilitating the release of dopamine and, like cocaine, by blocking dopamine uptake. Amphetamine-induced locomotor activity was not significantly different between groups (total crossover counts for controls: 892.35 $\pm$ 177.51; experimentals: 948.75 $\pm$ 217.51), $F(1,12) < 1$. When these animals were subsequently challenged with cocaine (10 days after the last boost), both measures of locomotor activity were again suppressed in experimental animals as compared with controls ($F(1,12) = 14.8$, $P < 0.002$). To investigate the basis for this blunted behavioural response, animals were killed 30 min after receiving the cocaine injection and their brains extracted for analysis. Levels of cocaine were found to be 52% lower in the striatal tissue and 77% lower in cerebellar

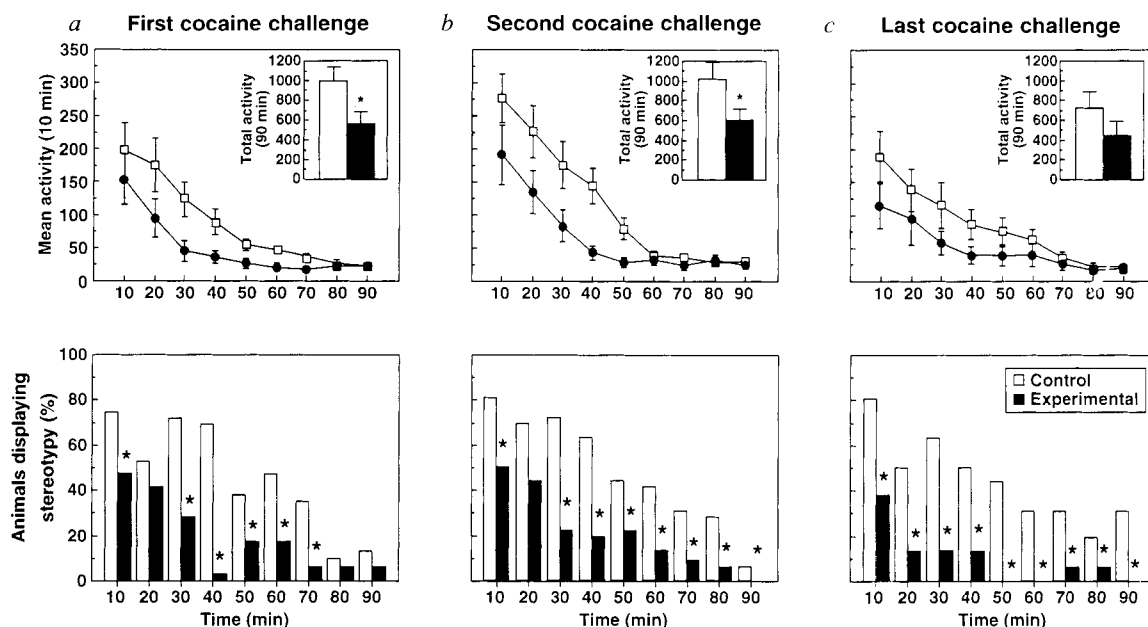


FIG. 3 Locomotor activity (crossovers) and stereotyped behaviour (sniffing and rearing) following intraperitoneal injection of cocaine post-immunization. Immunizations consisted of a 400 μ l bolus intraperitoneal (i.p.) injection of a RIBI adjuvant (MPL + TDM) (RIBI Immunochem Research) containing 250 μ g GNC-KLH or KLH in 100 mM PB, pH 7.4. The last boost was administered without adjuvant. The figure shows the response to post-immunization cocaine challenge on the third (a), seventh (b) and tenth (c) day following the last immunization boost. Locomotor activity was measured in photocell cages as in ref. 22. After a 90 min period of habituation, animals received an i.p. injection of 15 mg kg⁻¹ cocaine HCl mixed in saline solution (bolus 1 ml kg⁻¹) and their locomotor responses measured during a 90 min session. Based on locomotor activity scores, animals were assigned to the experimental or control group in ranking order. Locomotor data were analysed by subjecting 10-minute total means for locomotor activity to a two-factor analysis of variance (ANOVA) (group $\times$ time) with repeated measures

on the within-group factor, time. Stereotyped behaviour was rated as previously described²³. Data were analysed by a likelihood ratio method, the 'information statistic'^{24,25}. These figures depict the pooled data from two identical studies for a total of $n = 16$. At the time of the first cocaine challenge, there was a significant difference between control and experimental groups in both mean activity (a, top, control: 987.44 ± 149.5 ; experimental: 555.07 ± 124.7 ; $F(1,30) = 4.13$) and stereotypy (a, bottom, $2I = 85.25$, d.f. = 1.9). Both psychomotor measures were again significantly different at the time of the second cocaine challenge (locomotor (b, top, control: $1,035.19 \pm 152.92$; experimental: 598.25 ± 121.46); $F(1,30) = 3.97$) and stereotypy (b, bottom, $2I = 94.12$, d.f. = 1.9). By the last cocaine challenge, locomotor activity did not differ significantly between groups (c, top, control: 719.23 ± 133.77 ; experimental: 443.24 ± 199.50) whereas there was a significant difference in stereotypy (c, bottom, $2I = 85.25$, d.f. = 1.9); $*P < 0.05$, for difference between control group and experimental group.

tissue of the immunized animals compared to controls (Fig. 4).

In the first group of animals, the reduced psychomotor response observed during the first challenge persisted after subsequent cocaine injections in the experimental group (Fig. 3). At the second cocaine challenge, locomotor activity and stereotyped behaviour were significantly decreased in the experimental group (Fig. 3). At the third cocaine challenge, locomotor activity did not differ significantly between the control and experimental groups. However, there continued to be a highly significant difference in the amount of stereotyped behaviour. The decrease in stereotyped behaviour but not locomotor activity compared to controls may reflect the change in profile of psychomotor stimulation associated with repeated cocaine exposure. Typically, in control animals, repeated administration of cocaine shifts the cocaine dose-response curve to the left (sensitization) and this is reflected in less locomotor activity but more stereotyped behaviour¹². There was some evidence of an increased psychomotor response and a prolonged stereotyped behavioural response in the control group but this sensitization did not occur in the immunized animals (Fig. 3). However, it is important to emphasize that the overall psychomotor response to cocaine remained dramatically blunted in the immunized animals. These findings suggest that the level of cocaine reaching critical sites of action in the central nervous system of immunized rats was decreased at the time of post-immunization challenges, thus producing a markedly diminished psychomotor effect characteristic of lower doses of cocaine¹¹. This hypothesis is supported by the maintenance of antibody titre which was the same before the

first cocaine challenge and 8 days after the last cocaine challenge, a span of 25 days. Given enough time between cocaine injections, saturation of the immune mechanism may be avoided and protection may be sustained.

Analysis of cocaine concentrations in cerebral tissue revealed considerably lower levels of cocaine in the brains of immunized animals compared to those of controls after cocaine injection. This finding is compatible with the diminished psychostimulant activity observed in the immunized group. Moreover, there is a good correlation between the estimated levels of bound cocaine in the bloodstream and the difference found in the brains of experimental and control animals. In this regard, it seems that extreme antibody affinities are not necessary, but only that they match the peak concentrations of cocaine. It is possible that the thermodynamics of interaction between cocaine binding to antibodies and receptors may be an optimum^{18,19}. In this case, the *in vivo* immunoglobulin concentration can be a controlling factor in neutralizing the action of the drug. Importantly, the results are an indication that modulation of cocaine levels in the circulation directly influence behaviour. This may have implications regarding human administration in that peak plasma concentrations parallel maximum subjective effects²⁰. However, the effects of this type of vaccination protocol on the human condition ('binge-like' patterns) are difficult to estimate at this time and will require additional study.

Motor behaviour provides a sensitive and reproducible measure of drug action in rats under standardized conditions. This model was useful in the detection of immune-mediated

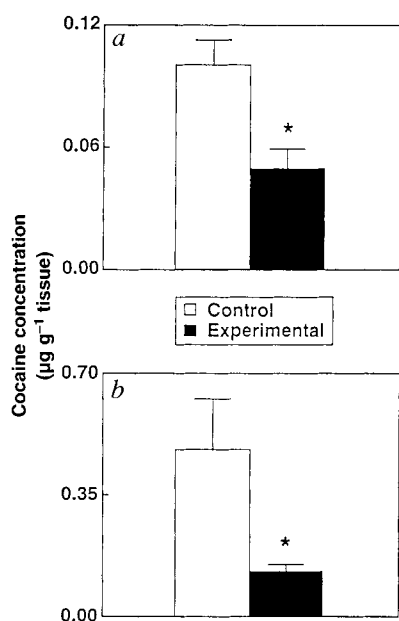


FIG. 4 Brain cocaine levels 30 min after i.p. cocaine injection. Cocaine concentrations were measured by reversed-phase HPLC¹⁷ on brain tissue from animals tested as before (10 days after last boost) except that locomotor activity was tested for only 30 min post-injection, at which time they were killed by decapitation. Their brains were rapidly removed and the striata and cerebellum dissected as previously described²⁶. Immediately following dissection, the striata and cerebellar samples from each animal were weighed, then frozen at -70°C for 3 days before cocaine extraction. Cocaine and its metabolites were extracted from brain tissue as described elsewhere¹⁷. Briefly, ~ 210 mg tissue was sonicated with 300 μl acetonitrile then centrifuged. The supernatant was decanted and cocaine and its metabolites were extracted into 700 μl chloroform:ethanol (4:1) and 70 μl of 0.1 M NaHCO_3 . Appropriate tissue samples for standards were prepared and analysed as in ref. 17. The cocaine levels measured correspond to free cocaine because cocaine-binding antibodies do not cross the blood-brain barrier, as shown by chromatographic separation and ultraviolet absorption spectra. Data were analysed by one-factor ANOVA. *a*, Striatal cocaine concentrations in experimental animals were significantly lower than those of control animals ($F(1,12)=10.37$; $P<0.01$). *b*, Cerebellar tissue was sampled to test the generality of cocaine distribution. Cerebellar cocaine concentrations in experimental animals were significantly lower than those of control animals ($F(1,12)=7.30$; $P<0.05$; * $P<0.05$). Data from two animals in the control group were not included in the subsequent cocaine-locomotor analysis because of two inadequate cocaine injections which resulted in a seizure ($n=1$), a limp hindlimb ($n=1$). Final group size: control $n=6$, experimental $n=8$.

blockade of cocaine-induced hypermotor effects. The results warrant further investigation of the potential for the immune-mediated response to alter reinforcing actions of cocaine in the context of drug-dependence models. Clearly, the surmountability of the antibody-induced blockade with higher doses of ingested cocaine will have to be explored. Furthermore, additional studies are needed to elucidate the efficacy of multiple immunizations. Elaboration of the current approach to include appropriate modifications of immunochemistry, manipulation of the immune system or incorporation of catalysis²¹ will perhaps prove advantageous. Because immunization therapy would exert its actions outside the central nervous system, it should have none of the side effects of conventional pharmacotherapy. In addition, such a protocol could be implemented as a prophylactic treatment and in relapse prevention. Thus, immunopharmacotherapy may offer a non-toxic, substance-specific strategy that should not affect normal neurochemical physiology, presenting a solid scientific approach for cocaine abuse treatment. □

Received 6 July; accepted 3 November 1995.

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ACKNOWLEDGEMENTS. We thank T. Danon and D. Kubitz for technical assistance with monoclonal and polyclonal antibodies, and R. A. Lerner, L. A. Gold and B. A. Baldo for comments on the manuscript. This research was supported by grants from the National Institute of Drug Abuse (K.D.J. and G.F.K.) and the Alfred P. Sloan Foundation (K.D.J.).

Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium channel

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THE peptide Phe-Met-Arg-Phe-NH₂ (FMRFamide) and structurally related peptides are present both in invertebrate and vertebrate nervous systems^{1,2}. Although they constitute a major class of invertebrate peptide neurotransmitters³, the molecular structure of their receptors has not yet been identified. In neurons of the snail *Helix aspersa*⁴, as well as in *Aplysia* bursting⁵ and motor⁶ neurons, FMRFamide induces a fast excitatory depolarizing response due to direct activation of an amiloride-sensitive Na⁺ channel⁴. We have now isolated a complementary DNA from *Helix* nervous tissue; when expressed in *Xenopus* oocytes, it encodes an FMRFamide-activated Na⁺ channel (FaNaCh) that can be blocked by amiloride. The corresponding protein shares a very low sequence identity with the previously cloned epithelial Na⁺ channel subunits^{7–12} and *Caenorhabditis elegans* degenerins^{13–15}, but it displays the same overall structural organization. To our knowledge, this is the first characterization of a peptide-gated ionotropic receptor.

The amiloride-sensitive Na⁺ channel which is activated by FMRFamide in *Helix aspersa* neurons⁴ shares pharmacological and biophysical properties with the recently cloned epithelial Na⁺ channel (ENaCh)^{7–12}. A 310-base pair (bp) cDNA fragment was amplified from *Helix* neurons with degenerate oligonucleotides designed from the known members of the ENaCh/

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Na^+ over K^+ ($P_{\text{Na}^+}/P_{\text{K}^+} > 10$) (Fig. 4c) and a higher conductance for Na^+ (13.1 ± 0.4 pS, in 96 mM NaCl, $n = 11$;) than for Li^+ (6.1 ± 0.1 pS, in 96 mM LiCl, $n = 3$). Channel activity is characterized by very brief openings and closings of the order of ms (Fig. 4b, e), by a slight increase in the open probability at nega-

tive potentials (Fig. 4d) and by very rare openings lasting several seconds (Fig. 4b, -70 mV lower trace).

These biophysical properties are all similar, if not identical, to those of the channel previously characterized directly in *Helix* neurons⁴. They are also similar to those of a mutated epithelial

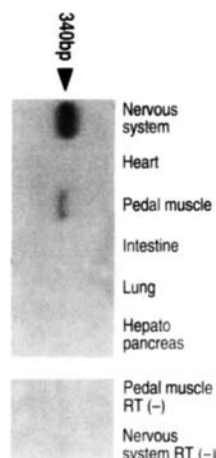


FIG. 2 Southern blot analysis of RT-PCR products for FaNaCh mRNA. A band of 340 bp (corresponding to the cDNA fragment located between nucleotides 1,493 and 1,833) was detected in nervous system and pedal muscle; RT(-), reaction without reverse transcriptase.

METHODS. cDNAs were synthesized from total RNA of *Helix* organs with MMLV reverse transcriptase (BRL) and oligo(dT) primer. Oligonucleotides used as primers for RT-PCR were located at position 1,493 bp (5'-ACCTCATCCGTCAGAACATG-3') and 1,813 bp (5'-CCAGCTGAGTCGGAGTAGTG-3') of the FaNaCh cDNA. PCR was carried out for 30 s at 94 °C, 1 min at 60 °C, and 1 min at 72 °C for 25 cycles. PCR products were separated on a 2% agarose gel, transferred to Hybond-N⁺ (Amersham), and hybridized using a [³²P]dCTP-labelled *Pst*I (base 971)-*Sty*I (base, 2,264) fragment of the entire FaNaCh cDNA.

FIG. 3 Macroscopic properties of the FMRFamide-activated current. **A**, Inward currents measured at -70 mV induced by brief (a) or continuous (b) applications of 30 μM FMRFamide. **c**, Inhibition of the FMRFamide-activated current by 100 μM amiloride (AMI). **d**, FMRFamide-activated current with the I544S mutant. As for wild-type FaNaCh, EDPFLRFamide (30 μM) failed to activate the mutated channel. **B**, Dose-response curves for FMRFamide and FLRFamide; i/i_{max} is the amplitude of the FMRFamide or FLRFamide-activated current divided by the maximal response. Each point represents mean values from 6 to 8 oocytes. **C**, Mean I-V relationships measured by voltage ramps from -150 to +100 mV, before and during application of 30 μM FMRFamide on 5 oocytes. **D**, Inhibition of the FMRFamide-activated current (30 μM) measured at -70 mV by amiloride, benzamil, and EIPA; each point represents mean values from 7 oocytes. **E**, Ionic dependence of the FMRFamide-activated current (30 μM) for the wild type and the I544S mutant; mean control current was measured in 10 oocytes for the wild type and 15 oocytes for the I544S mutant in a buffer containing (in mM): NaCl 96, KCl 2, MgCl₂ 1, CaCl₂ 1.8, HEPES 5, pH 7.4. Asterisk indicates a significant difference to wild-type FaNaCh with $P < 0.05$ using an unpaired t-test.

METHODS. Oocytes were injected with 1.5 ng to 50 ng cRNA, as described¹⁸. For macroscopic current measurements, FMRFamide or related peptides (Sigma, Peninsula) were briefly applied in the experiment chamber by a buffer pipette (100 μl).

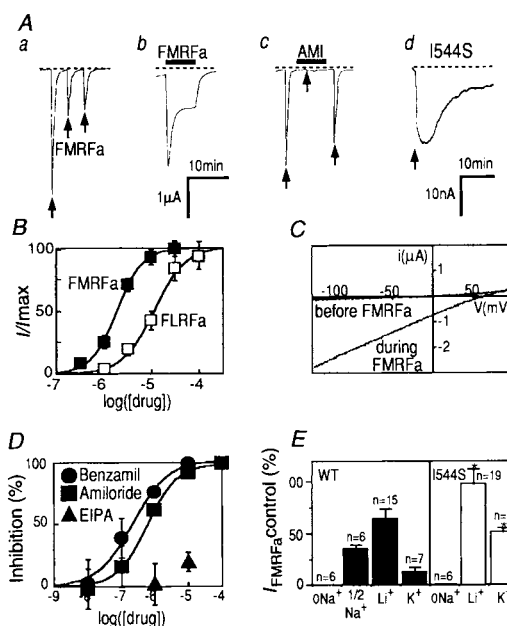
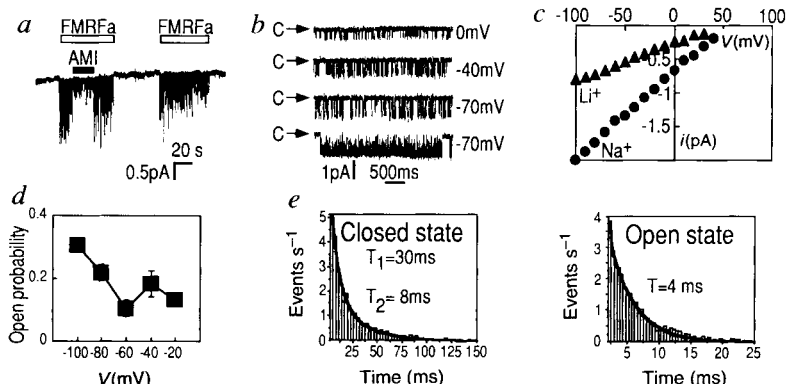


FIG. 4 Single-channel properties of the FMRFamide-activated channel. **a**, Unitary currents induced by FMRFamide (30 μM) recorded from an outside-out patch held at -80 mV; 100 μM of amiloride (AMI) was used to block the activated current. **b**, Cell-attached recordings at various membrane potentials with 30 μM FMRFamide in the pipette solution. **c**, Mean I-V relationships with Na^+ or Li^+ in the pipette. **d**, Voltage dependence of the open probability measured in 6 different patches. **e**, Open and closed time histograms at -60 mV; data were collected from 4 different patches.

METHODS. Cell-attached recordings were performed on oocytes clamped to zero mV in high K^+ medium. Pipettes contained (in mM): NaCl (or LiCl) 140, MgCl₂ 1, CaCl₂ 1, HEPES 10, pH 7.4. For outside-out patches pipettes contained (in mM): KCl 140, MgCl₂ 2, EGTA 5, HEPES 10, pH 7.4 and bath contained Na^+ -rich solution (see above). Data were filtered at 1 kHz for analysis. Open probabilities and mean dwell times were calculated with Biopatch software (Biologic). Values are means $\pm$ s.e.m.



Na⁺ channel in which Ser 589 in the α -subunit has been replaced by an isoleucine¹⁸, that is, the equivalent residue found in FaNaCh and Mec-4 sequences. Ile 544 of FaNaCh is located in the highly conserved region ($\geq 60\%$ identity with ENaCh and degenerins between amino acids 540 and 551 of FaNaCh) known to be critical in the formation of the ionic pore of ENaCh¹⁸. Mutation of Ile 544 into a serine in FaNaCh changed activation/inactivation kinetics of the Na⁺ current induced by FMRFamide (Fig. 3*Ad*), increased the channel permeability for Li⁺ and K⁺ versus Na⁺ (Fig. 3*E*) and rendered the channel insensitive to 100 μ M amiloride ($n=8$, data not shown).

The FaNaCh channel which is involved in neurotransmission, is clearly a new member of a channel superfamily including the epithelial Na⁺ channel involved in transcellular Na⁺ transport and Na⁺ homeostasis^{26,27} as well as in taste perception²⁸, and the degenerins, involved in mechanotransduction^{13,15}, probably by forming mechanosensitive channels and which can be mutated to cause neuronal degeneration^{13,15}.

Neuropeptides are generally considered to modify cellular electrical activity by binding to G-protein-coupled receptors capable of indirect modification of ion channel activity after stimulation of a second messenger cascade. The FaNaCh channel is the first cloned ionic channel which is directly gated by a peptide. This suggests that gated channels may also exist for other peptides. In light of the presence of FMRFamide-like peptides in the mammalian nervous system and their strong connections to the opiate system²⁹, studies should be undertaken to identify the mammalian counterpart(s) of the *Helix* FaNaCh channel. $\square$

A possible docking and fusion particle for synaptic transmission

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SEVERAL proteins have been implicated in the rapid (millisecond) calcium-controlled release of transmitters at nerve endings^{1,2}, including soluble *N*-ethylmaleimide-sensitive fusion protein (NSF³⁻⁵) and soluble NSF attachment protein (α -SNAP^{3,6}), the synaptic SNAP receptor (SNARE)^{3,7} and the calcium-binding protein synaptotagmin², which may function as a calcium sensor in exocytosis⁸. A second SNAP isoform (β -SNAP), which is 83% identical to α -SNAP, is highly expressed in brain⁹, but its role is still unclear. Here we show that these proteins assemble cooperatively to form a docking and fusion complex. β -SNAP (but not α -SNAP) binds synaptotagmin and recruits NSF, indicating that the complex may link the process of membrane fusion to calcium entry by attaching a specialized fusion protein (β -SNAP) to a calcium sensor (synaptotagmin). Polyphosphoinositols that block transmitter release, inositol 1,3,4,5-tetrakisphosphate (InsP₄), inositol 1,3,4,5,6-pentakisphosphate (InsP₅) and inositol 1,2,3,4,5,6-hexakisphosphate (InsP₆), also block the assembly of the particle by preventing β -SNAP from binding to synaptotagmin.

On addition of α - or β -SNAP to octylglucoside extracts of brain membranes, only α -SNAP was recovered bound to the SNAREs (results not shown). As either SNAP isoform can bind to isolated SNAREs (Fig. 1*c*), β -SNAP, unlike α -SNAP, must

Received 18 July; accepted 10 October 1995.

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ACKNOWLEDGEMENTS. We are grateful to M. Crest for providing the snail organs, to G. Romey, N. Voilley, R. Waldmann and F. Bassilana for fruitful discussions and to M. Bordes, N. Leroudier, C. Roulinat, A. Douy and F. Aguila for expert technical assistance. This work was supported by the Centre National de la Recherche Scientifique (CNRS) and the Association Française de Lutte contre la Mucoviscidose (AFLM).

bind with high affinity to an abundant membrane protein in crude extracts. A polypeptide of relative molecular mass 65,000 (*M*_r 65K) from crude extracts specifically bound by glutathione *S*-transferase (GST) β -SNAP fusion protein was identified as synaptotagmin (tagmin) by western blotting (results not shown).

Beads containing GST linked to the tagmin I cytoplasmic domain (residues 79–421; Fig. 1*a*) bind about 1 mol of β -SNAP per mol of tagmin at saturation (Fig. 1*b*, lane 3), but neither α -SNAP nor the other ubiquitous SNAP isoform, γ -SNAP⁹, bind (lane 2, and results not shown). Binding activity is confined to the carboxy-terminal portion of tagmin containing the C2B domain (residues 248–421; Fig. 1*b*, lane 9); the amino-terminal portion containing the C2A domain (residues 96–265) which binds calcium and acidic phospholipids¹⁰ is not involved (lane 8).

Because NSF binds to membranes in a SNAP-dependent manner^{11,12}, we tested whether NSF might bind to the β -SNAP/tagmin complex. Binding was indeed efficient (Fig. 1*b*, lane 5), and α -SNAP neither substituted for β -SNAP (lane 4) nor interfered with binding (lane 7).

NSF that is bound to SNAREs through α -SNAP hydrolyses ATP, releasing NSF and α -SNAP and disrupting the SNARE complex⁷. Complexes of NSF bound to tagmin through β -SNAP, however, are stable in the presence of Mg²⁺ ATP with (Fig. 1*b*, lane 13) or without (lane 12) 0.3 mM calcium, and their formation is unaffected by calcium (results not shown). The lack of effect of Mg²⁺-ATP indicates that the binding of β -SNAP and NSF differs from that seen in 20S particles^{3,7}, and implies that the tagmin/ β -SNAP/NSF 'triple' complex should be stable *in vivo* even when ample ATP is present. Tagaya and colleagues¹³ have reported that NSF on synaptic vesicles is not released by Mg²⁺ ATP; the tagmin/ β -SNAP/NSF triple complex may explain their findings.

Could the triple complex contribute to synaptic vesicle docking? If so, tagmin's role would be superimposed on the general role of SNAREs in docking, as previously suggested^{14,15} (a possibility supported by the lack of release of vesicles when

the vesicle SNARE (v-SNARE), vesicle-associated membrane protein (VAMP) or the target membrane SNARE (t-SNARE), syntaxin, is absent^{16,17}). Assembled SNARE complexes indeed bind to triple complex immobilized on beads, but only when α -SNAP is also added (Fig. 2, lanes 7, 8), underscoring the specificity of the interaction. β -SNAP could substitute for α -SNAP but is less effective (lane 9). β -SNAP's affinity for SNAREs was indeed two- to threefold lower than that of α -SNAP (Fig. 1c); similar amounts of each are bound at saturation (about 3 mols per SNAP-25). All three SNAREs (VAMP, syntaxin and SNAP-25) bind together (lane 8), along with α -SNAP (not shown); the previously associated β -SNAP and NSF remain bound (not shown). Beads containing just GST-tagmin, or GST-tagmin/ β -SNAP complexes, did not support efficient binding of α -SNAP/SNARE complexes (Fig. 2), which required NSF and was not significantly affected by calcium (not shown).

We reported previously⁷ that endogenous tagmin bound to the native synaptic SNARE complex could be displaced by excess α -SNAP, suggesting that the SNARE complex could not simultaneously bind both tagmin and NSF/SNAPs. The cytoplasmic domain of recombinant tagmin also binds weakly to the SNARE complex (Fig. 2, lane 1) and is displaced by excess α -SNAP. Thus, our new data do not contradict, but rather extend, the previous findings by showing that the highly specific interaction

of tagmin with β -SNAP allows the SNARE complex simultaneously to bind tagmin, β -SNAP and NSF.

As physiological^{6,18}, pathophysiological¹⁹ or genetic tests^{5,8,16} indicate that each of the particle's subunits or a close homologue is required for exocytosis *in vivo*, this complex probably represents a specialized docking and fusion particle for regulated exocytosis at the synapse, and perhaps elsewhere.

Recently, Mikoshiba and co-workers²⁰ showed that the C2B domain of tagmin binds the polyphosphoinositols InsP_4 , InsP_5 and InsP_6 , but not inositol 1,4,5-trisphosphate (InsP_3). As the C2B domain also binds β -SNAP (Fig. 1b, lane 9), we tested the effects of these compounds on this interaction (Fig. 3). The rank order and potency of the effect of the compounds was the same as that of their binding to tagmin²⁰. Preformed tagmin/ β -SNAP and tagmin/ β -SNAP/NSF triple complexes were unaffected, however (results not shown), indicating that active polyphosphoinositols introduced *in vivo* would only block the assembly of new triple complexes and the docking and fusion particles formed from them. Polyphosphoinositols do indeed block neurotransmission on presynaptic injection, with a lag time of 15–45 min²¹. Although this may reflect the effects of these compounds on the binding of β -SNAP to tagmin, additional effects cannot be excluded.

NSF bound in the 'triple' complex with β -SNAP and tagmin could hydrolyse ATP to release VAMP on entering the possible

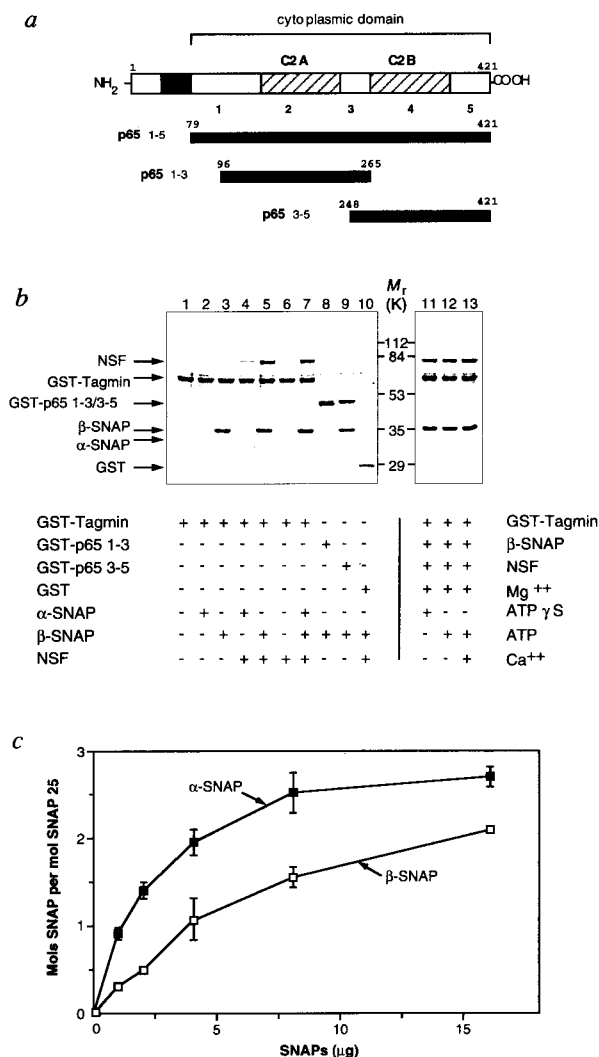


FIG. 1 Binding specificity of α -SNAP and β -SNAP for SNAREs and to tagmin. a, Schematic representation of synaptotagmin I and its fragments (hatched boxes, C2 domains; solid box, transmembrane region). b, Specific binding of β -SNAP to tagmin and to its C-terminal part and the subsequent recruitment of NSF (left panel). Tagmin- β -SNAP-NSF complex is stable in the presence of Ca^{2+} - Mg^{2+} -ATP (right panel). GST fusion proteins of different tagmin constructs or GST alone were incubated with α -SNAP (lanes 2, 4, 7) or β -SNAP (lanes 3, 5, 7-13) with (lanes 4-7, 10-13) or without (lanes 1-3, 8, 9) addition of NSF. Three samples, identical to that in lane 5, were further incubated in the presence of ATP- γ S (lane 11), ATP (lane 12) or ATP + Ca^{2+} (lane 13). c, α -SNAP and β -SNAP binding to the SNARE complex. SNARE complexes were isolated from bovine brain detergent extract and incubated with increasing amounts of α -SNAP (■) or β -SNAP (□).

METHODS. GST fusion proteins containing the cytoplasmic domain of synaptotagmin I (amino acids 79-421; 4 μ g), the N-terminal (GST-p65₁₋₃; amino acids 96-265; 3 μ g) or the C-terminal (GST-p65₃₋₅; amino acids 248-421; 3 μ g) parts, were immobilized on GSH-agarose beads. Then 3 μ g of His₆- α -SNAP³ or 3 μ g of His₆- β -SNAP were diluted in 250 μ l of 20 mM HEPES-KOH, pH 7.6, 150 mM potassium acetate, 1% glycerol, 5 μ M EDTA, 0.8% (w/v) octyl- β -D-glucopyranoside, 0.4 mg ml⁻¹ ovalbumin, spun at 15,000g for 5 min and the supernatant was incubated with beads for 1 h at 4 °C. The beads were then washed twice with 250 μ l of the same buffer without ovalbumin. Some samples were further incubated for 1 h at 4 °C with 5 μ g of recombinant NSF³ in 250 μ l of 20 mM HEPES-KOH, pH 7.6, 150 mM potassium acetate, 1 mM MgCl₂, 5 μ M EGTA, 0.5 mM ADP, 1% glycerol, 0.8% (w/v) octyl- β -D-glucopyranoside, 0.4 mg ml⁻¹ ovalbumin. The beads were then washed in the same buffer without ovalbumin and samples analysed in SDS-PAGE. The stability of the complex were tested by incubating the complex-bound beads for 30 min at 37 °C in 20 mM HEPES-KOH, pH 7.6, 150 mM potassium acetate, 1 mM MgCl₂, 1% glycerol, 0.5% (w/v) Triton X-100 (buffer A) containing 0.5 mM ATP- γ S/1 mM EGTA, 0.5 mM ATP/1 mM EGTA, or 0.5 mM ATP/300 μ M free Ca^{2+} . For determination of the α / β -SNAP binding to the SNARE complex, increasing amounts of SNAPs were diluted in 20 mM HEPES-KOH, pH 7.6, 150 mM potassium acetate, 1% glycerol, 0.5% (w/v) Triton X-100 (buffer B), pre-spun and added to beads containing the SNARE complex isolated from bovine brain extract (250 μ g of protein per sample), by using a monoclonal anti-syntaxin antibody coupled to Protein G Fast Flow⁷. After 1 h at 4 °C the beads were isolated, washed and the samples analysed by Tris-urea/SDS-PAGE³. The amount of recovered proteins was evaluated by densitometric scanning of the Coomassie Blue R-250 stained bands. Data points represent the average of three independent experiments.

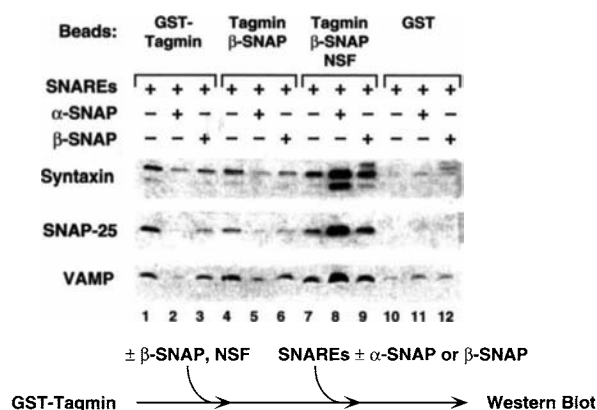


FIG. 2 Requirements for the assembly of the specialized docking and fusion particle. Immobilized GST-tagmin/β-SNAP/NSF complexes and GST were incubated with bovine brain detergent extracts in the absence or presence of α-SNAP or β-SNAP. Bound syntaxin 1A and 1B (upper panel), SNAP-25 (middle panel) and VAMP (lower panel) were analysed by immunodecoration of western blots.

METHODS. GST, GST-tagmin, GST-tagmin/β-SNAP and GST-tagmin/β-SNAP/NSF complexes, bound to GSH-agarose beads were prepared as described for Fig. 1. The complexes were resuspended in 20 mM HEPES-KOH, pH 7.6, 150 mM potassium acetate, 1% glycerol, 0.8% (w/v) octyl-β-D-glucopyranoside, (buffer C) containing 1 mM MgCl₂, 0.5 mM ADP, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol and mixed in a final volume of 500 μl with bovine brain extract (250 μg of protein) prepared as described³ by using octyl-β-D-glucopyranoside as detergent. His₆-α-SNAP (10 μg) or 10 μg of His₆-β-SNAP was added as indicated. The samples were incubated for 1 h at 4 °C, recovered by centrifugation at 5,000g for 1 min and washed three times in buffer C containing 1 mM MgCl₂, 0.5 mM ADP. The pellets were analysed by Tris-urea/SDS-PAGE, transferred to nitrocellulose and immunodecorated with a monoclonal antibody against syntaxin/HPC1 and with polyclonal antibodies against SNAP-25 and against rat VAMP⁷. Immunoreactive bands were visualized by using the Enhanced Chemoluminescence method (ECL; Amersham). Similar results were obtained using Triton X-100-treated bovine membrane extract³ and by controlling the free calcium concentration to 1 μM as described for calcium-dependent priming²³.

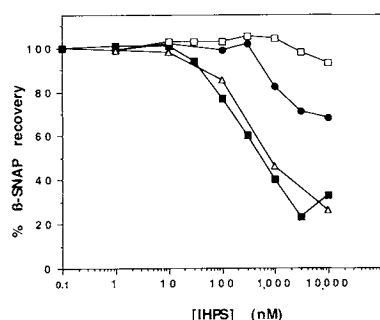


FIG. 3 Effect of polyphosphoinositols (IHPS) on the binding of β-SNAP to tagmin. Immobilized GST-tagmin was incubated with β-SNAP in the presence of different polyphosphoinositols at increasing concentrations. The results were expressed as percentage of the amount of β-SNAP bound to tagmin in the absence of polyphosphoinositols. ■, InsP₃; △, InsP₅; ●, InsP₄; □, InsP₆; data represent the average of three to five independent experiments.

METHODS. GST-tagmin (4 μg) immobilized on GSH-agarose beads were incubated for 15 min at 4 °C with variable concentrations of InsP₃, InsP₄, InsP₅ or InsP₆ (Sigma). Then β-SNAP (4 μg) diluted in buffer C containing 0.4 mg ml⁻¹ ovalbumin and previously spun at 15,000g for 5 min was added. The samples (250 μl final volume) were incubated for 1 h at 4 °C, washed twice with 250 μl of buffer C and then analysed by SDS-PAGE. The β-SNAP bound to GST-tagmin was determined by scanning of the Coomassie Blue R250 stained gel as described previously (see Fig. 1 legend).

docking and fusion particle, as shown by cleavage by tetanus toxin²² (Fig. 4). ATP-γS did not substitute for ATP, and a hydrolysis-deficient mutant of NSF (E329Q/D604Q double mutant)¹¹ permitted particle assembly, but produced particles resistant to Mg²⁺-ATP (results not shown). The new docking and fusion particle is therefore not a dead-end complex.

Calcium did not affect the ATP-dependent disassembly of the particle (Fig. 4). Studies of calcium-triggered exocytosis in patch-clamped and in permeabilized neuroendocrine cells show that a slow, ATP-dependent but calcium-independent process, termed vesicle priming, is followed by a rapid ATP-independent step triggered by calcium^{23, 25}.

Our observations indicate that priming may include the disruption of the proposed synaptic docking and fusion particle by the NSF-ATPase, producing a latent fusogenically active state awaiting calcium. If this is so, disruption of the docking and fusion particle should, like priming, be independent of calcium, as we observed (Fig. 4). This view in no way contradicts the additional need for ATP during the priming stage to maintain the pool of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) required for fusion after calcium activation²⁶. The protein components constituting the postulated fusogenically active state produced once ATP is hydrolysed by NSF are unknown. However, this complex should contain both the calcium regulator and a fusogenic protein(s). The special affinity of tagmin for β-SNAP may therefore be significant.

Our observations also suggest that synaptotagmin should be regarded as a specialized v-SNARE, as well as a calcium sensor.

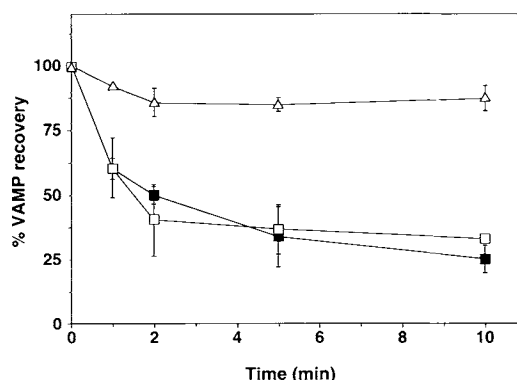


FIG. 4 Mg²⁺-ATP-dependent disassembly of the specialized docking and fusion particle. Immobilized complexes were treated in conditions that allow Mg²⁺-ATP-dependent SNARE complex disassembly. This process was monitored by detecting the exposure of VAMP to tetanus toxin following disruption of the SNARE complex (△, samples plus Mg²⁺-ATP-γS; □, samples plus Mg²⁺-ATP; ■, samples plus Mg²⁺-ATP and Ca²⁺). The same kinetics were observed when 10 times higher levels of toxin than stated below were used, implying that toxin attack was not rate limiting.

METHODS. The complex of GST-tagmin/β-SNAP/NSF with SNAREs and α-SNAP was assembled from Triton X-100-treated bovine brain membrane extract³ as described for Figs 1 and 2 and washed twice with buffer B containing 1 mM MgCl₂ and 0.5 mM ADP. The particle-containing beads (15 μl per sample) were then added to 235 μl of pre-warmed buffer A supplemented with 400 nM of the recombinant catalytic subunit of tetanus toxin²² and containing, alternatively, 0.5 mM ATP-γS/1 mM EGTA, 0.5 mM ATP/1 mM EGTA, or 0.5 mM ATP/300 μM free Ca²⁺ (obtained by buffering with 1 mM EGTA). At the indicated times, samples were chilled on ice and centrifuged at 5,000g for 1 min. Pellets were analysed by Tris-urea/SDS-PAGE, transferred to nitrocellulose and immunodecorated with a polyclonal antibody against rat VAMP⁷. Immunoreactive bands were visualized by ECL and quantified by scanning the resulting X-ray film. VAMP content at different incubation times was expressed as a percentage of the amount present at time zero. Analysis of supernatants showed only trace amounts of VAMP, independent of the time of incubation, as expected (not shown).

Like other v-SNAREs⁵, it is present in transport vesicles, binds a t-SNARE (syntaxin)¹⁴, is a specialized SNAP receptor (binding β - but not α -SNAP), enters a docking and fusion particle and is released when NSF hydrolyses ATP. □

Received 28 July; accepted 20 October 1995.

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ACKNOWLEDGEMENTS. We thank R. Scheller for the gift of tagmin constructs, anti-VAMP and anti-tagmin antibodies, H. Niemann for providing an expression clone encoding tetanus toxin light chain, and W. Eng and N. Arango for technical assistance. This work was supported by an NIH grant to J.E.R. and by the Mathers Charitable Foundation. Fellowship support came from EMBO (to G.S.), Max Kade Foundation, Inc. (M.J.S.G.) and Deutsche Forschungsgemeinschaft (G.S.).

Apoptosis of T cells mediated by galectin-1

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GALACTIN-1, a member of the family of β -galactoside binding proteins¹, has growth regulatory and immunomodulatory activities^{2–4}. We report here that galectin-1, expressed by stromal cells in human thymus and lymph nodes^{5,6}, is present at sites of cell death by apoptosis during normal T-cell development and maturation. Galectin-1 induced apoptosis of activated human T cells and human T leukaemia cell lines. Resting T cells also bound galectin-1, but did not undergo apoptosis. Human endothelial cells that expressed galectin-1 induced apoptosis of bound T cells. Galectin-1-induced apoptosis required expression of CD45, and was decreased when N-glycan elongation was blocked by treatment of the cells by swainsonine⁷, whereas inhibition of O-glycan elongation⁸ potentiated the apoptotic effect of galectin-1. Induction of apoptosis by an endogenous mammalian lectin represents a new mechanism for regulating the immune response.

Galectin-1^{9–13} mediates cell–cell and cell–substrate interactions of normal and neoplastic cells^{5,14–16}. Galectin-1 had therapeutic activity against autoimmune disease in two animal models^{3,4}; in one study, galectin-1 treatment resulted in decreased numbers of antigen-reactive T cells⁴, suggesting that the immunomodulatory activity resulted from the direct deletion of reactive T cells. As galectin-1 is expressed in peripheral lymphoid tissue^{6,17}, we asked whether galectin-1 would induce apoptosis of T cells.

Recombinant human galectin-1 induced apoptosis of activated human T cells^{18,19}. Membrane blebbing, margination of chromatin and cell shrinkage were visible within 90 min after galectin-1 treatment, and apoptotic bodies appeared within 5 h. The number of cells with hypodiploid DNA content was increased in samples treated with galectin-1 (Fig. 1a, b), and DNA fragmentation was detected by *in situ* nick end-labelling (Fig. 1c, d).

Bound galectin-1 was removed from T cells by the addition of lactose after exposure times of 0 to 360 min. Exposure of T cells to galectin-1 for 30 min resulted in an irreversible commit-

ment to apoptosis (Fig. 1e), whereas cells exposed to galectin-1 for 10 min or less had no increase in cell death compared to controls. These results demonstrated that inhibition of galactose-specific interactions prevented galectin-1-induced apoptosis, and that an irreversible event occurred between 10 and 30 min after the exposure of T cells to galectin-1.

Galectin-1 has no transmembrane domain, and is a homodimer with a dissociation constant (K_d) of $7 \mu\text{M}$ ²⁰. Dimeric galectin-1 remains cell-surface associated^{5,20}, and has been proposed to bridge opposing glycoproteins^{14–16, 20, 21}. As galectin-1 can bind multiple lactosamine units on an oligosaccharide chain, dimeric galectin-1 may also crosslink cell surface counter-receptors. Dose–response analysis demonstrated that $10 \mu\text{M}$ galectin-1 was required to induce T-cell apoptosis (Fig. 1f), indicating that the dimeric form of galectin-1 was necessary for induction of apoptosis. We did not detect apoptosis of these cells when treated with galectin-3 ($20 \mu\text{M}$), which has a similar saccharide specificity but exists primarily as a monomer¹⁰. As galectin-1 can constitute up to 1% of the total protein in cells that synthesize it¹⁵, galectin-1 on the cell surface is likely to be present in the dimeric form.

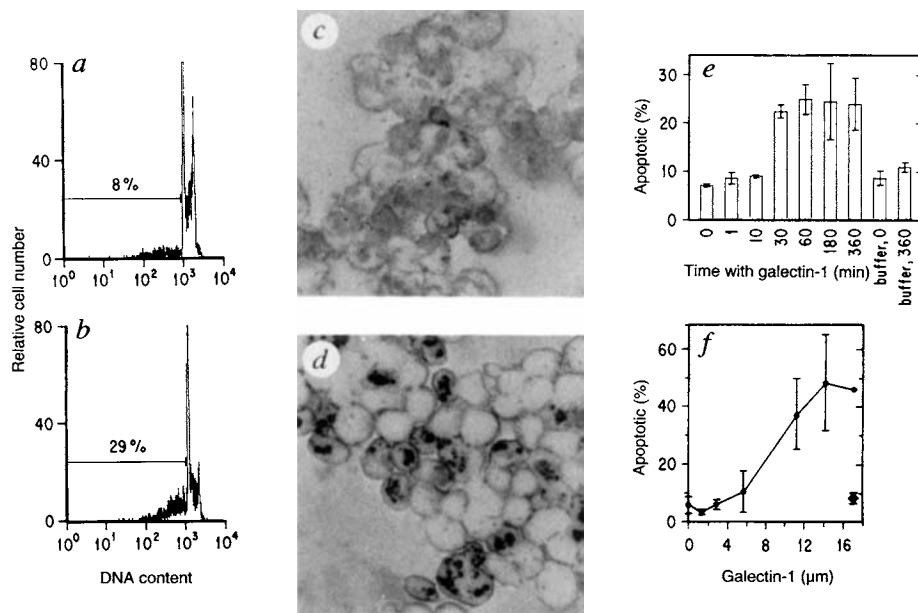
Primary cultures of human aortic endothelial cells (HAEC) express galectin-1 on the cell surface, and treatment of HAECs with minimally oxidized low-density lipoprotein (MM-LDL) increased expression of galectin-1⁶. To examine whether cells that make galectin-1 can kill T cells, we investigated the ability of HAEC to induce apoptosis. HAEC were incubated with or without MM-LDL before the addition of MOLT-4 cells for 4 h. The same number of MOLT-4 cells bound to control and MM-LDL-treated HAEC (34.5 ± 12 and 31.8 ± 12.4 , respectively; high power field). However, a twofold increase in apoptosis was detected when T cells were bound to MM-LDL-treated HAEC, with increased cell surface galectin-1 (Fig. 2a, b). These data demonstrate that endothelial cells which express galectin-1, can induce apoptosis of susceptible T cells.

The apoptotic effect of soluble galectin-1 depended upon the activation state of the T cells. Although both resting and activated T cells bound galectin-1, only activated T cells underwent apoptosis when exposed to galectin-1 (Fig. 2c, d). 35% of activated T cells, but only 7% of peripheral blood mononuclear cells (PBMs) underwent apoptosis in response to galectin-1, a response similar to that reported for treatment with anti-CD3²².

We tested human T leukaemia cell lines for the ability to bind galectin-1 and for susceptibility to galectin-1-mediated apoptosis (Fig. 2e). Each of the cell lines bound comparable levels of biotinylated-galectin-1 (not shown). However, the cell lines varied in sensitivity to galectin-1. The CD3⁺ cell line ARR (C. Uittenbogaart, personal communication) was susceptible to galectin-1. CEM cells were not susceptible to galectin-1, in con-

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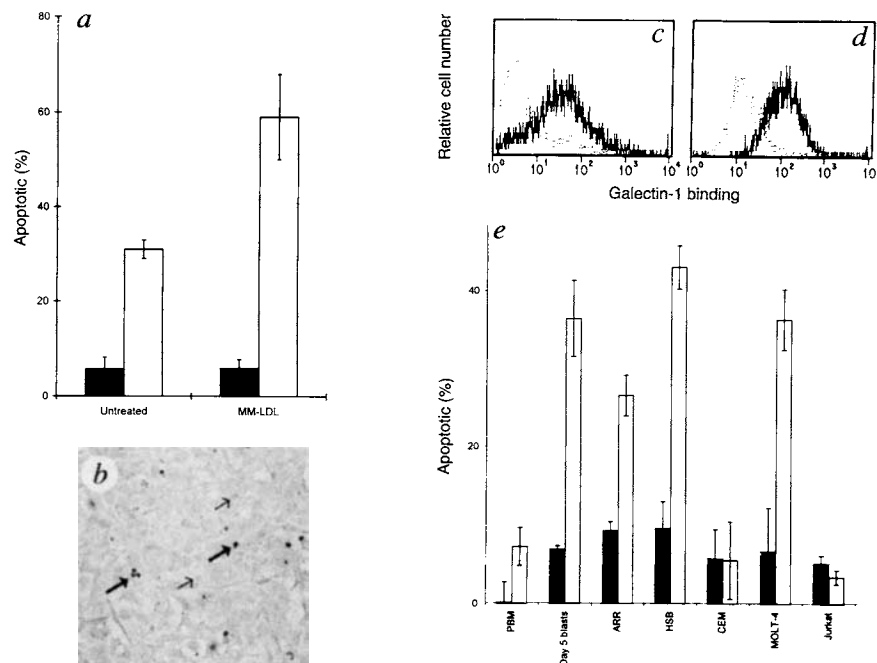
FIG. 1. Galectin-1 induces apoptosis of T cells through carbohydrate-specific interactions. **a, b**, Galectin-1 treatment of day-5 PHA blasts induced DNA fragmentation detected as an increase in the per cent of cells with hypodiploid DNA content¹⁹ in galectin-1-treated (**b**) relative to untreated (**a**) cells. **c, d**, Galectin-1 also induced DNA fragmentation of MOLT-4 T lymphoblastoid cells as detected by hypodiploid DNA content (not shown), and by *in situ* nick end-labelling³⁰. **e**, Apoptosis was reversed by the addition of lactose up to 10 min after exposure to galectin-1. **f**, 10 μ M galectin-1 was necessary to induce apoptosis, suggesting a requirement for the dimeric form of galectin-1. **METHODS.** **a, b**, Human PBM cells were cultured with 8 μ g ml⁻¹ phytohaemagglutinin (PHA, Pharmacia) in complete medium (RPMI 1640, 10 mM HEPES, 10% fetal bovine serum) at 37 °C. Day-5 blasts were centrifuged over Ficoll-Paque (Pharmacia) to enrich for viable cells, then cultured for 8 h at 37 °C in complete medium with 3 mM dithiothreitol (DTT, Sigma Chemical Co.), with (b) or without, (a) 16 μ M human galectin-1 (ref. 5). Samples were adjusted to 0.1 M lactose, washed in phosphate buffered saline (PBS), fixed and stained¹⁹ with 7-aminoactinomycin D (7-AAD, Molecular Probes) before analysis on a Becton Dickinson FACSCAN. Numbers indicate the percentage of cells with hypodiploid DNA content. **c, d**, MOLT-4 cells (cultured to 10^5 cells per ml), centrifuged over Ficoll-Paque, were incubated as above in the presence (d) or absence (c) of 20 μ M galectin-1 for 5.25 h at 37 °C. Samples were adjusted to 0.1 M lactose. Cytospin preparations, fixed in methanol, were incubated for 1 h at 37 °C with 2 pmols μ l⁻¹ digoxigenin-dUTP (Boehringer Mannheim) and 2.0 units μ l⁻¹ terminal deoxynucleotidyl transferase (Promega) according to the manufacturer's directions. Incorporation of digoxigenin-dUTP was detected using sheep anti-digoxigenin Fab coupled to alkaline phosphatase (Boehringer Mannheim) and NBT/BCIP as substrates



(GibcoBRL). Magnification $\times 400$. **e**, Day-5 PHA blasts were exposed to 14 μ M galectin-1 for the indicated times before galectin-1 was removed by 0.1 M lactose. Cells were incubated at 37 °C in medium for a total of 7 h. The percentage of apoptotic cells was determined by morphological analysis after staining with 7-AAD. Over 200 cells from each sample were scored as apoptotic or non-apoptotic by a blinded observer. Data are the mean $\pm$ s.d. of duplicate determinations from two experiments. **f**, Day-5 PHA blasts were cultured in varying concentrations of galectin-1 ($\bullet$), or heat-inactivated galectin-1 ($\blacklozenge$) for 7.5 h at 37 °C. Apoptosis was determined as in **e**.

FIG. 2 **a, b**, Human endothelial cells expressing galectin-1 induce apoptosis of T lymphoblastoid cells. Human aortic endothelial cells (HAECs) were treated with buffer alone (untreated) or with minimally oxidized LDL (MM-LDL). Untreated and treated endothelial cells bound equal numbers of MOLT-4 cells per well (data not shown). However, the percentage of apoptotic T cells (**a**, unshaded) bound to the endothelial cells was increased when the endothelial cells were treated with MM-LDL, which increases cell surface expression of galectin-1⁶. The percentage of apoptotic T cells in wells without HAECs (black bars) was $\sim 5\%$ in all samples. **b**, MOLT-4 cells adhere to a HAEC monolayer and undergo apoptosis. Large arrows indicate apoptotic cells, stained by *in situ* nick end-labelling. Small arrows indicate non-apoptotic MOLT-4 cells. **c–e**, Both resting and activated T cells bound galectin-1, but only activated T cells underwent apoptosis in response to galectin-1. Five human T-cell lines tested bound comparable levels of biotin-galectin-1 (not shown); of these, ARR, MOLT-4 and HSB were susceptible to galectin-1-induced apoptosis, whereas Jurkat and CEM cells were not (**e**).

METHODS. Confluent monolayers of HAECs plated on 15-mm coverslips in M199 with 20% FBS were treated with MM-LDL (100 μ g ml⁻¹) or buffer (PBS, 0.01% EDTA, 10^{-5} M butylated hydroxytoluene) for 4 h at 37 °C. MOLT-4 cells (3.3×10^5 per well) were added to HAEC monolayers, or to control wells with and without MM-LDL, for 4 h at 37 °C. After removal of unbound cells by washing, the coverslips were fixed in 4% paraformaldehyde and stained by *in situ* nick end-labelling as in Fig. 1c, d. Cytospin preparations were made from control wells and similarly stained. The number of adherent MOLT-4 cells and the percentage of labelled apoptotic cells were determined by light microscope examination. Twenty-four high-power fields were counted for each sample, and the percentage of apoptotic cells was calculated as the mean value $\pm$ s.d. from a representative of two experiments. **c**, PBM, or **d**, day-5 PHA blasts were



incubated on ice for 1 h with biotinylated human galectin-1 (14 μ M) in the presence ($\cdots$) or absence ($—$) of 0.1 M lactose to inhibit specific binding. After washing, cells were stained with phycoerythrin-conjugated streptavidin (Molecular Probes) and analysed by flow cytometry on a Becton-Dickinson FACSCAN. **e**, Day-5 PHA blasts, PBMs and human T-cell lines were cultured to densities of 1.0 – 1.5×10^5 cells per ml, centrifuged over Ficoll-Paque, then incubated in the presence (unshaded) or absence (black) of 14 μ M galectin-1 for 7 h at 37 °C. The percentage of apoptotic cells was determined by morphological analysis after staining with 7-AAD. Error bars indicate the s.d. of triplicate determinations.

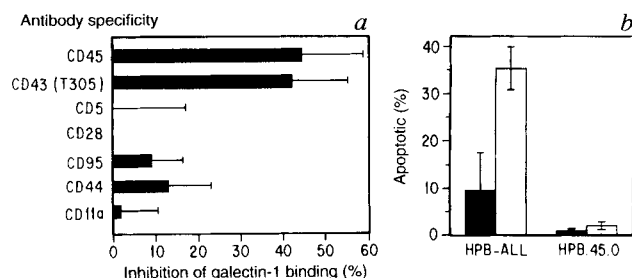


FIG. 3 CD45 participates in galectin-1-mediated apoptosis. *a*, Antibodies to CD43 and CD45 inhibited galectin-1 binding to MOLT-4 cells. *b*, Galectin-1 mediated apoptosis of HPB cells requires expression of CD45. Of the wild-type HPB-ALL cells, 35% underwent apoptosis in response to galectin-1. In contrast, galectin-1 did not induce apoptosis of the CD45 negative variant.

METHODS. *a*, Monoclonal antibodies were preincubated with MOLT-4 cells in PBSA, 1% bovine serum albumin for 30 min at 4 °C in a 96-well plate. Biotinylated galectin-1 was added to a final concentration of 0.7 μ M and the plates were incubated for 1 h at 4 °C. After washing, cells were incubated with horseradish peroxidase-conjugated streptavidin (Zymed, So. San Francisco, CA). Cell-associated peroxidase activity was detected by ELISA, using the colorimetric substrate *o*-phenylenediamine dihydrochloride. Results are expressed as mean $\pm$ s.d. of triplicate wells. Antibodies were obtained from the following sources: T305 (CD43) the gift of R. Fox (Scripps Clinic and Research Foundation); LCA (CD45) Dako Corp.; MHM4 (CD11a), and IOL44 (CD44) AMAC, Inc.; T1 (CD5) Coulter; DX2 (CD95) PharMingen; Leu-28 (CD28) Becton-Dickinson. *b*, The T-cell lines HPB-ALL and HPB.45.0, a CD45 negative variant of the line, were incubated in complete medium, 3 mM DTT at 10×10^6 cells per ml in the presence (unshaded) and absence (black) of 16 μ M human galectin-1. After 5.25 h at 37 °C, the cells were fixed in ethanol and the percentage of apoptotic cells was determined by morphological analysis after staining with 7-AAD. Results are expressed as the mean $\pm$ s.d. of triplicate samples from a representative of three experiments. HPB-ALL and HPB.45.0 cells were the gift of A. Weiss (Univ. of California, San Francisco).

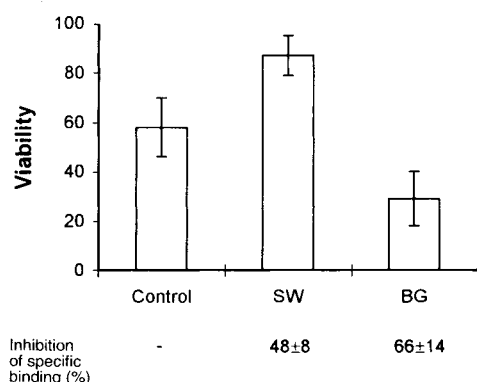


FIG. 4 Susceptibility of activated T cells to galectin-1 was altered by pre-incubation with inhibitors of glycosylation. Swainsonine (SW) inhibited mannosidase II and N-linked oligosaccharide processing⁷. Benzyl- α -GalNAc (BG) inhibits O-linked oligosaccharide chain elongation⁸. SW treatment abrogated galectin-1-induced cell death, whereas BG treatment potentiated it. Both SW and BG decreased the lactose-inhibitable binding of galectin-1.

METHODS. PBM were cultured for 24 h with PHA, then cultured for an additional 65 h with either 5 μ g ml⁻¹ SW, 2 mM BG, or a buffer control. Galectin-1 binding and viability were determined by flow cytometric analysis. Galectin-1 binding was determined as in Fig. 2. The relative fluorescence of each sample was determined as (mean fluorescence channel_{galectin-1} - mean fluorescence channel_{galectin-1 + lactose}) / Δ mfc. The percentage inhibition of specific binding was determined as (Δ mfc inhibitor treated $\div$ Δ mfc control) $\times$ 100. In each sample, dead cells were identified by the uptake of 7-AAD. The percentage change in viability is shown as the mean $\pm$ s.d. from two separate experiments.

trast to reports that CEM cells are sensitive to both anti-Fas and corticosteroid-induced apoptosis^{23,24}, although MOLT-4 cells, which are resistant to anti-Fas²⁴, were very sensitive to galectin-1. The mechanism of galectin-1-induced apoptosis thus appears to be distinct from that triggered by Fas or CD3 engagement.

To identify candidate counter-receptors for galectin-1, we used an antibody inhibition assay. Antibodies to leukosialin (CD43) and leukocyte common antigen (CD45) significantly inhibited galectin-1 binding to MOLT-4 cells (Fig. 3*a*). CD43 and CD45 are T-cell counter-receptors for galectin-1 expressed by thymic epithelial cells⁵. Antibodies to CD45 also blocked galectin-1-induced apoptosis (not shown), whereas antibody treatment alone did not induce apoptosis. Anti-CD95 (Fas)²⁵, did not inhibit the binding of galectin-1, nor did antibodies against CD5, CD11a, CD28 and CD44.

CD45 has been proposed to participate in apoptosis²⁶. We examined the ability of galectin-1 to induce apoptosis of the human T-cell line HPB-ALL and a CD45⁻ variant of this cell line, HPB.45.0 (ref. 27). Both HPB-ALL and HPB.45.0 agglutinated upon addition of galectin-1. However, HPB-ALL underwent apoptosis when treated with galectin-1, whereas the CD45⁻ HPB.45.0 did not (Fig. 3*b*).

CD45 exists in a number of isoforms, which are differently glycosylated²⁸. The core of CD45, present on all isoforms, bears 11 *N*-glycans. Higher molecular mass forms of CD45, present on naive peripheral T cells, contain *O*-glycosylated amino-terminal domains. Both *N*- and *O*-glycans on CD45 can contain the polylactosamine sequence preferentially recognized by galectin-1. We investigated the relative contributions of *N*- and *O*-glycans to galectin-1-induced cell death by culturing activated T-cell blasts with swainsonine, to inhibit *N*-glycan processing⁷, or benzyl- α -GalNAc, to inhibit *O*-glycan elongation⁸. Swainsonine treatment decreased the binding of galectin-1 to T cells, and reduced galectin-1-induced cell death (Fig. 4). Swainsonine treatment did not affect the level of expression of CD45 or CD43 (not shown). Benzyl- α -GalNAc treatment also decreased galectin-1 binding. Unlike swainsonine, benzyl- α -GalNAc potentiated the cytotoxic effect of galectin-1, resulting in an increase in galectin-1-induced cell death. These results indicate that *N*-glycans participate in galectin-1-induced apoptosis, and suggest that *O*-glycans may modulate the effect of galectin-1, by masking *N*-glycans involved in the apoptotic signal²⁹. Although unstimulated PBMs expressed the CD45RA isoform, activated T cells expressed both CD45RO and CD45RA isoforms (not shown). As CD45RO lacks the *O*-glycan-rich N-terminal domains, CD45RO molecules on activated T cells may have *N*-glycans that are more accessible to galectin-1.

Our data demonstrate that human galectin-1 induces apoptosis of activated T cells, a new function for a mammalian lectin. These findings suggest a mechanism for the observed immunosuppressive effects of galectin-1 in animal models of autoimmune disease. Because galectin-1 has been found in tumours such as ovarian carcinomas¹⁵, as well as in inflamed human lymphoid tissues⁶, galectin-1 may modulate the immune response in a variety of pathological processes. □

Received 14 September; accepted 12 October 1995.

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ACKNOWLEDGEMENTS. We thank A. Weiss for providing us with HPB-ALL and HPB.45.0 cell lines, D. Cooper for galectin-3, J. Berliner for assistance with HAEC cultures, J. Braun, R. Childs, W. R. Clark, T. Feizi, C. Uittenbogaart and O. Witte for their critical reading of the manuscript, A. Arvand, D. Boehr, A. Delageane, A. Patrikian, T. Wu and the staff of the UCLA Jonsson Comprehensive Cancer Center flow cytometry core laboratory for technical assistance. This work was supported by a grant from the UCLA Frontiers in Science Project and an award from the University of California Cancer Research Coordinating committee to L.G.B., by NRSA training grants to N.L.P., and an NIH grant to the Jonsson Comprehensive Cancer Center.

Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast

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IN fission yeast the onset of mitosis is brought about by Cdc2/Cdc13 kinase, which is inhibited by the Wee1/Mik1 tyrosine kinases and activated by Cdc25 tyrosine phosphatase¹. This control network integrates many signals, including those that monitor DNA replication, DNA damage and cell size. We report here that a fission yeast MAP kinase pathway links the cell-cycle G₂/M control with changes in the extracellular environment that affect cell physiology. Fission yeast *spc1*⁻ mutants have a G₂ delay that is greatly exacerbated by growth in high osmolarity media and nutrient limitation. A lethal interaction of *spc1* and *cdc25* mutations shows that Spc1 promotes the onset of mitosis. Spc1 is a MAP kinase homologue that is activated by Wis1 kinase in response to osmotic stress and nutrient limitation. Spc1 is inactivated by Pyp1, a phosphatase previously identified as a mitotic inhibitor^{2,3}. Pyp1 dephosphorylates only tyrosine-173 of Spc1, unlike the dual-specificity phosphatases that have been shown to regulate other MAP kinases⁴.

Schizosaccharomyces pombe *spc1*⁻ mutations were first identified as recessive suppressors of lethality caused by loss of protein phosphatase 2C (PP2C) activity⁵. These mutations also conferred sensitivity to high osmolarity medium. A closer examination revealed that the *spc1*⁻ osmosensitive phenotype involved a cell-cycle defect (Fig. 1). A *spc1-M13* mutant grew well in nutrient-rich YES medium at 30 °C, although it was somewhat elongated. About 50% of the *spc1-M13* cells lysed in YES + 1 M KCl, but the other cells became highly elongated, undergoing cell-cycle arrest (Fig. 1a). Identical results were observed with YES + 1.5 M sorbitol medium. The *spc1-M13* cell elongation phenotype was also accentuated in EMM2 minimal medium (Fig. 1b). The difference between YES and EMM2 was cancelled by the addition of yeast extract nutrients to EMM2 (Fig. 1b), showing that the accentuated cell-cycle delay was caused by nutrient limitation.

Flow cytometry showed that *spc1-M13* cells have a 2C DNA content, indicating a cell-cycle delay at G₂/M (data not shown). This possibility was explored by crossing the temperature-sensitive *cdc25-22* mutation into a *spc1-M13* background. We found that *spc1-M13 cdc25-22* cells were inviable at permissive temperature (Fig. 1c), showing that *spc1-M13* cells have a defect in promoting mitosis. Importantly, *spc1-M13 cdc25-22 weel-50* triple mutant cells also underwent cell-cycle arrest at 35 °C (Fig. 1d), showing that Spc1 can function independently of Wee1 and Cdc25. Mutational inactivation of Mik1, a second kinase that inhibits Cdc2 (ref. 6), caused no phenotype in a *spc1-M13* background (data not shown), indicating that Spc1 does not regulate Mik1.

spc1⁺ was cloned by rescue of *spc1-M13* osmosensitivity. Disruption of *spc1*⁺ confirmed that *spc1-M13* is a null allele. *spc1*⁺ encodes a roughly 40K MAP kinase (MAPK) homologue (Fig. 2). The Thr 171 and Tyr 173 residues are in comparable positions in all MAPKs; MAPK kinases activate MAPKs by phosphorylating these residues^{7,9}. Spc1 is most similar to *Saccharomyces cerevisiae* Hog1 (80% identity), murine p38, human CSBP, *Xenopus* Mpk2 (~50% identity) and human JNK1 (38% identity)^{10–14}. Hog1, p38 and Mpk2 are stimulated in response to osmotic stress^{10,12,13}.

Immunoblot analysis showed that Spc1 is tyrosine phosphorylated in cells grown in YES (Fig. 3a). This indicates that Spc1 is active in YES, consistent with the elongated phenotype of *spc1-M13* mutants grown in YES. Growth in YES containing 0.4 M NaCl or 0.4 M KCl caused a rapid increase of Spc1 tyrosine phosphorylation (Fig. 3a). The signal was also increased in cells cultured in EMM2 (Fig. 3a), indicating that Spc1 is activated by the same conditions that accentuate the G₂/M delay in *spc1-M13* cells.

Four types of evidence suggested that Spc1 was likely to be activated by Wis1 kinase¹⁵. First *wis1*⁻ (*spc2*⁻) mutations were identified in the same genetic screen that yields *spc1*⁻ mutations⁵. Second, *wis1*⁻ and *spc1-M13* mutants exhibited identical phenotypes in high osmolarity YES and in EMM2 medium (data not shown). Third, these phenotypes were not enhanced in *spc1*⁻ *wis1*⁻ double mutants, suggesting that Spc1 and Wis1 function in a linear pathway. Fourth, it was observed that high overexpression of *wis1*⁺ was toxic in a wild-type background, causing a cell swelling and lysis phenotype (Fig. 3b, lower left panel), whereas the morphology of *spc1-M13* cells was unaffected by *wis1*⁺ overexpression (lower right panel).

The Wis1/Spc1 relationship was investigated by expressing GST-Spc1 in wild-type and *Δwis1* cells (Fig. 3c, left panel). GST-Spc1 was tyrosine phosphorylated in wild-type but not in *Δwis1* cells (Fig. 3c, right panel). GST-Spc1 purified from wild-type cells phosphorylated myelin basic protein (MBP), whereas GST-Spc1 from *Δwis1* cells was inactive (Fig. 3c, lower panel). We next examined whether Wis1 activates Spc1 *in vitro*. Inactive, non-tyrosine phosphorylated GST-Spc1 was purified from *Δwis1* cells and incubated with purified Wis1. Wis1 caused both the tyrosine phosphorylation and activation of GST-Spc1 (Fig. 3d).

Discovery of the Wis1/Spc1 pathway brought to mind earlier studies that identified two tyrosine phosphatases, Pyp1 and Pyp2, as mitotic inhibitors^{2,3}. Pyp1 or Pyp2 overexpression caused a G₂/M delay, whereas *Δpyp1* caused advancement of M. The hypothesis that Spc1 is a Pyp1 substrate was supported by finding that the G₂/M delay caused by Pyp1 overproduction was exacerbated in high osmolarity medium (Fig. 3e). Additional support for the model was provided by finding that neither overexpression or disruption of *pyp1*⁺ or *pyp2*⁺ had any effect in a *spc1-M13* background (data not shown). We therefore examined whether Pyp1 modulated Spc1 tyrosine phosphorylation *in vivo*. Immunoblotting showed that GST-Spc1 tyrosine phosphorylation was increased ~fivefold in *Δpyp1* cells and abolished in Pyp1 overproducers (Fig. 4a). Experiments were then carried out to determine whether Spc1 and Pyp1 interacted directly *in*

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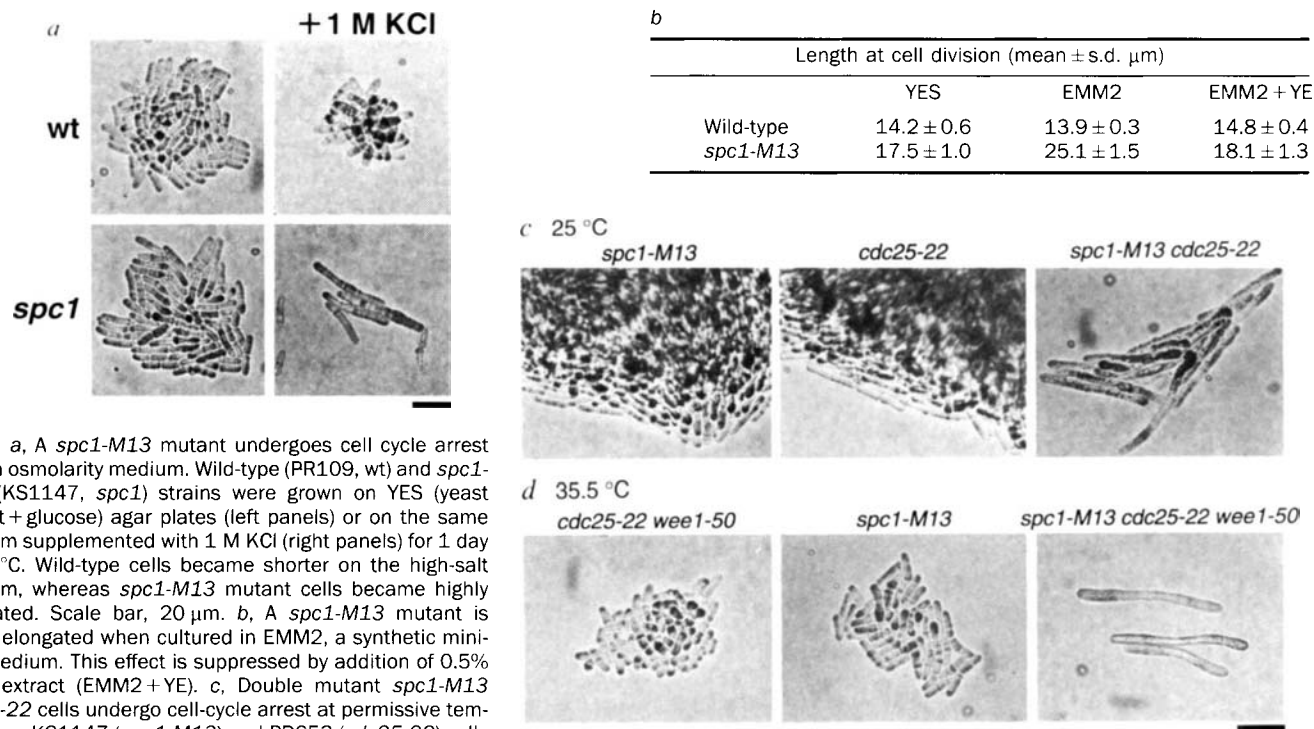


FIG. 1 *a*, A *spc1-M13* mutant undergoes cell cycle arrest in high osmolarity medium. Wild-type (PR109, wt) and *spc1-M13* (KS1147, *spc1*) strains were grown on YES (yeast extract + glucose) agar plates (left panels) or on the same medium supplemented with 1 M KCl (right panels) for 1 day at 30 °C. Wild-type cells became shorter on the high-salt medium, whereas *spc1-M13* mutant cells became highly elongated. Scale bar, 20 μ m. *b*, A *spc1-M13* mutant is highly elongated when cultured in EMM2, a synthetic minimal medium. This effect is suppressed by addition of 0.5% yeast extract (EMM2 + YE). *c*, Double mutant *spc1-M13 cdc25-22* cells undergo cell-cycle arrest at permissive temperature. KS1147 (*spc1-M13*) and PR653 (*cdc25-22*) cells were mated and asci dissected on YES medium at 25 °C. Single mutants were elongated but grew well, whereas *spc1-M13 cdc25-22* double mutant cells underwent 3–4 divisions and then arrested as highly elongated cells. *d*, Strains KS1315 (*spc1-M13 wee1-50*) and KS1361 (*cdc25-22 wee1-50*) were mated and viable triple mutants were isolated on YES at 25 °C. KS1361 (*cdc25-22 wee1-50*) cells were viable at 35.5 °C on YES medium, dividing with semi-wee phenotype, whereas a triple mutant strain KS1374 (*spc1-M13 cdc25-22 wee1-50*) underwent a cdc arrest at 35.5 °C on YES. At 35.5 °C

KS1147 (*spc1-M13*) cells appeared moderately elongated relative to wild-type. The *cdc25-22* and *wee1-50* alleles are conditional temperature sensitive; *spc1-M13* is not conditional. Scale bar, 20 μ m. METHODS. Media and procedures for studying fission yeast have been described²³. Cell length measurements were made using an eyepiece micrometer with cells grown to mid-log phase in liquid medium. Cell length at division is measured as mean $\pm$ s.d. in μ m. Twenty septated cells were measured in each case.

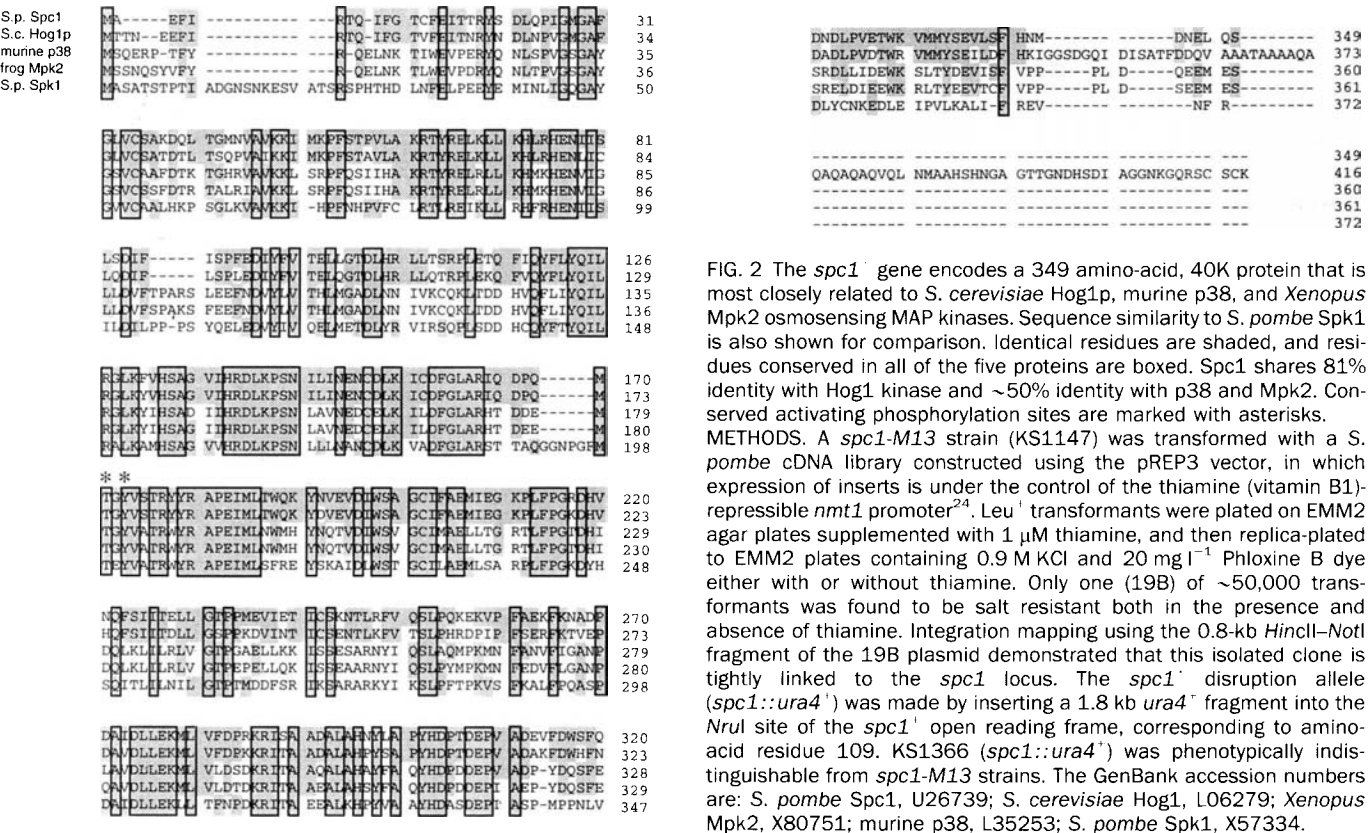


FIG. 2 The *spc1* gene encodes a 349 amino-acid, 40K protein that is most closely related to *S. cerevisiae* Hog1p, murine p38, and *Xenopus* Mpk2 osmosensing MAP kinases. Sequence similarity to *S. pombe* Spk1 is also shown for comparison. Identical residues are shaded, and residues conserved in all of the five proteins are boxed. Spc1 shares 81% identity with Hog1 kinase and ~50% identity with p38 and Mpk2. Conserved activating phosphorylation sites are marked with asterisks. METHODS. A *spc1-M13* strain (KS1147) was transformed with a *S. pombe* cDNA library constructed using the pREP3 vector, in which expression of inserts is under the control of the thiamine (vitamin B1)-repressible *nmt1* promoter²⁴. Leu⁻ transformants were plated on EMM2 agar plates supplemented with 1 μ M thiamine, and then replica-plated to EMM2 plates containing 0.9 M KCl and 20 mg l⁻¹ Phloxine B dye either with or without thiamine. Only one (19B) of ~50,000 transformants was found to be salt resistant both in the presence and absence of thiamine. Integration mapping using the 0.8-kb *HincII*-*NotI* fragment of the 19B plasmid demonstrated that this isolated clone is tightly linked to the *spc1* locus. The *spc1* disruption allele (*spc1::ura4*⁺) was made by inserting a 1.8 kb *ura4*⁺ fragment into the *NruI* site of the *spc1* open reading frame, corresponding to amino-acid residue 109. KS1366 (*spc1::ura4*⁺) was phenotypically indistinguishable from *spc1-M13* strains. The GenBank accession numbers are: *S. pombe* Spc1, U26739; *S. cerevisiae* Hog1, L06279; *Xenopus* Mpk2, X80751; murine p38, L35253; *S. pombe* Spk1, X57334.

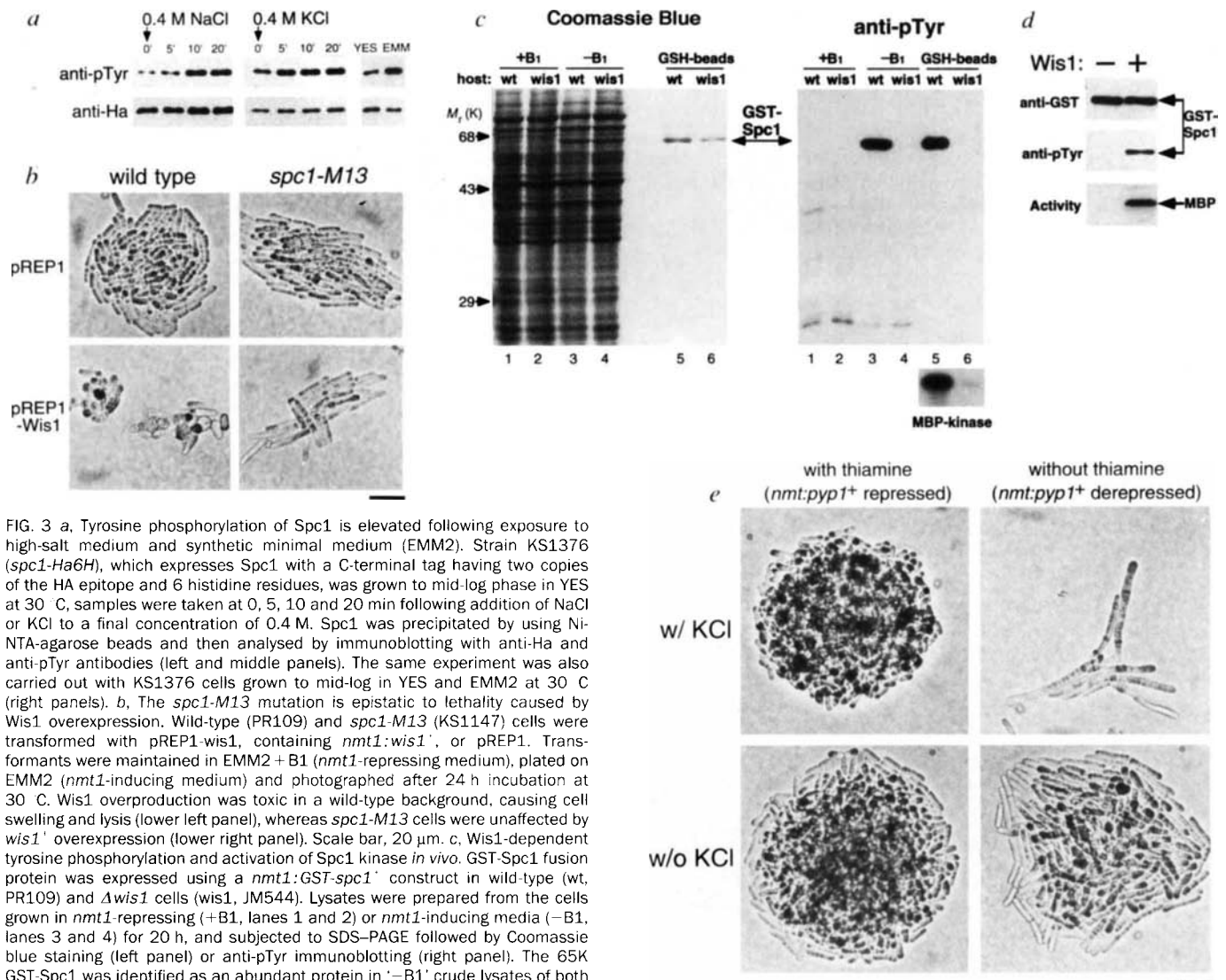


FIG. 3 *a*, Tyrosine phosphorylation of Spc1 is elevated following exposure to high-salt medium and synthetic minimal medium (EMM2). Strain KS1376 (*spc1-Ha6H*), which expresses Spc1 with a C-terminal tag having two copies of the HA epitope and 6 histidine residues, was grown to mid-log phase in YES at 30 °C, samples were taken at 0, 5, 10 and 20 min following addition of NaCl or KCl to a final concentration of 0.4 M. Spc1 was precipitated by using Ni-NTA-agarose beads and then analysed by immunoblotting with anti-Ha and anti-pTyr antibodies (left and middle panels). The same experiment was also carried out with KS1376 cells grown to mid-log in YES and EMM2 at 30 °C (right panels). *b*, The *spc1-M13* mutation is epistatic to lethality caused by Wis1 overexpression. Wild-type (PR109) and *spc1-M13* (KS1147) cells were transformed with pREP1-wis1, containing *nmt1::wis1*, or pREP1. Transformants were maintained in EMM2 + B1 (*nmt1*-repressing medium), plated on EMM2 (*nmt1*-inducing medium) and photographed after 24 h incubation at 30 °C. Wis1 overproduction was toxic in a wild-type background, causing cell swelling and lysis (lower left panel), whereas *spc1-M13* cells were unaffected by *wis1* overexpression (lower right panel). Scale bar, 20 µm. *c*, Wis1-dependent tyrosine phosphorylation and activation of Spc1 kinase *in vivo*. GST-Spc1 fusion protein was expressed using a *nmt1::GST-spc1* construct in wild-type (wt, PR109) and Δ *wis1* cells (*wis1*, JM544). Lysates were prepared from the cells grown in *nmt1*-repressing (+B1, lanes 1 and 2) or *nmt1*-inducing media (-B1, lanes 3 and 4) for 20 h, and subjected to SDS-PAGE followed by Coomassie blue staining (left panel) or anti-pTyr immunoblotting (right panel). The 65K GST-Spc1 was identified as an abundant protein in '-B1' crude lysates of both *wis1* and Δ *wis1* cells (lanes 3 and 4, left panel); but was only detected with anti-pTyr antibodies in *wis1* cells (lanes 3 and 4, right panel). GST-Spc1 was also purified from '-B1' lysates of wild-type and the Δ *wis1* mutant cells using GSH-Sepharose beads (left panel, lanes 5 and 6). Anti-pTyr immunoblotting of the purified GST-Spc1 confirmed that Spc1 tyrosine phosphorylation was dependent on Wis1 activity *in vivo* (right panel, lanes 5 and 6). The purified GST-Spc1 was also tested for kinase activity against myelin basic protein (lower right panel, MBP-kinase). This showed that Spc1 kinase activity was dependent on Wis1 *in vivo*. Separate experiments showed that expression of GST-Spc1 fully rescued the *spc1-M13* mutant phenotypes (data not shown). *d*, Wis1 tyrosine phosphorylates and activates GST-Spc1 *in vitro*. Inactive GST-Spc1 was purified from a Δ *wis1* strain and incubated in a kinase assay buffer containing ATP with Wis1 purified from a *spc1-M13* strain (right lane) or without Wis1 (left lane). Immunoblotting using anti-GST (upper panel) and anti-pTyr (middle panel) antibodies showed that Wis1 phosphorylated GST-Spc1 on tyrosine. The appearance of phosphotyrosine coincided with activation of GST-Spc1 kinase (lower panel). *e*, Pyp1-overproducer cells undergo cdc arrest in high-salt medium. Wild-type cells (PR109) transformed with a plasmid containing *nmt1::pyp1* (pREP1-Pyp1) were grown in EMM2 + 1 M KCl (top panels) or no added KCl (bottom panels). Overexpression of *pyp1* caused cell elongation in standard EMM2 (lower right panel) and cell-cycle arrest in EMM2 + KCl (upper right panel). The cdc-arrested cells had a single nucleus located at the centre of the cell. Note also that addition of KCl caused a reduction of cell size in cells that do not overproduce Pyp1 (upper left panel). Scale bar, 20 µm. **METHODS.** The *spc1* open reading frame (ORF) was amplified by PCR and cloned into a modified pREP1 vector, pREP1-Ha6H²⁵. The 1.1-kb *NdeI*-*Bam*HI fragment of the *spc1-Ha6H* fusion gene, lacking the *nmt1* promoter, was subcloned into a vector containing *S. pombe ura4* marker gene. The resultant plasmid was linearized at the *NruI* site in *spc1* and integrated into a wild-type strain (PR109). Integration was confirmed by Southern hybridization. This construct allowed Spc1 to be purified by Ni-NTA-agarose chromatography and detected by anti-Ha antibodies²⁵. This strain was indistinguishable from wild-type, indicating that epitope-tagged Spc1 was fully functional. Spc1-Ha6H protein was purified in denaturing conditions using Ni-NTA-agarose beads (Qiagen) following the manufacturer's protocol, then analysed by immunoblotting with

anti-Ha (12CA5) and anti-pTyr (4G10, Upstate Biotechnology) antibodies. For expression of GST-Spc1, PCR-amplified *spc1* was first cloned into the pGEX-KG vector²⁶, to construct pGEX-KG-*spc1*, and then GST-Spc1 was further subcloned behind the *nmt1* promoter in pREP1²⁴. Wild-type (PR109) and *wis1::ura4* (JM544) strains transformed with pREP1-GST-Spc1 were grown for 18 h in EMM2 medium to induce GST-Spc1 expression. Purification procedures were performed at 4 °C. Cells were disrupted with glass beads in L buffer (50 mM Tris-HCl pH 8.0, 400 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol, 50 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF) and the homogenate was centrifuged at 14,000g. The supernatant was diluted 1:1 with L buffer containing 0.2% NP-40 and incubated with 20 µl GSH-Sepharose (Pharmacia) for 1 h. The beads were washed three times with L buffer containing 0.1% NP-40 and subjected to either SDS-PAGE or MBP kinase assay²⁷. GST-Wis1 was purified from a *spc1-M13* strain carrying pREP1-GST-Wis1 plasmid (KS1384). Inactive GST-Spc1 was purified from a Δ *wis1* strain carrying pREP1-GST-Spc1 (KS1359). Lysate preparation, GSH-Sepharose absorption and washes were performed in L buffer containing 0.1% NP-40. Wis1 was cleaved from GST-Wis1 by incubation with thrombin (0.4 unit, Sigma) in T-buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, 0.1% 2-mercaptoethanol) for 20 min at 25 °C. Eluted Wis1 was mixed with GST-Spc1 bound to Sepharose beads. Phosphorylations were performed in P buffer (20 mM Tris-HCl pH 7.2, 10 mM MgCl₂, 1 mM MnCl₂, 0.1 mM EGTA, 0.1 mM Na₃VO₄, 1 mM 2-mercaptoethanol, 0.1 mg ml⁻¹ BSA) containing 100 µM ATP for 60 min at 25 °C. After extensive washes with L-buffer to remove Wis1, an aliquot was used for immunoblotting and the remainder was used for a MBP kinase assay. A *NdeI*-*NotI* fragment of *pyp1* was prepared by PCR and cloned into pREP1-Ha6H²⁵. Construction of pREP1-Pyp1(C470S) is described in Fig. 4. Cell size measurements were made with cells grown at 30 °C in YES. Strain designations are: PR109 (wild-type), KS1147 (*spc1-M13*), JM535 (*pyp1::LEU2*), PR355 (*pyp2::ura4*), KS1327 (*spc1-M13 pyp1::LEU2*), and KS1328 (*spc1-M13 pyp2::ura4*). In the cell size measurements involving overexpression of *pyp1* and *pyp1*-C470S, cells were grown at 30 °C in EMM2 for 20 h to induce the *nmt1* promoter. Plasmid pREP1-Pyp1 contains *nmt1::pyp1*, plasmid pREP1-Pyp1(C470S) contains *nmt1::pyp1*-C470S.

in vivo. Fission yeast cells expressing GST or GST-Spc1 were transformed with plasmids expressing Pyp1 or catalytically inactive Pyp1^{C470S}. Overexpression of Pyp1^{C470S} causes a G₂/M delay (data not shown), perhaps due to a stabilized inhibitory interaction with its substrate¹⁶. No Pyp1 copurified with GST, whereas Pyp1 was readily detected in the GST-Spc1 sample (Fig. 4b, lanes 1 and 2). A much larger amount of Pyp1^{C470S} protein coprecipitated with GST-Spc1 (Fig. 4b, lane 4), demonstrating that Spc1 is an *in vivo* substrate of Pyp1.

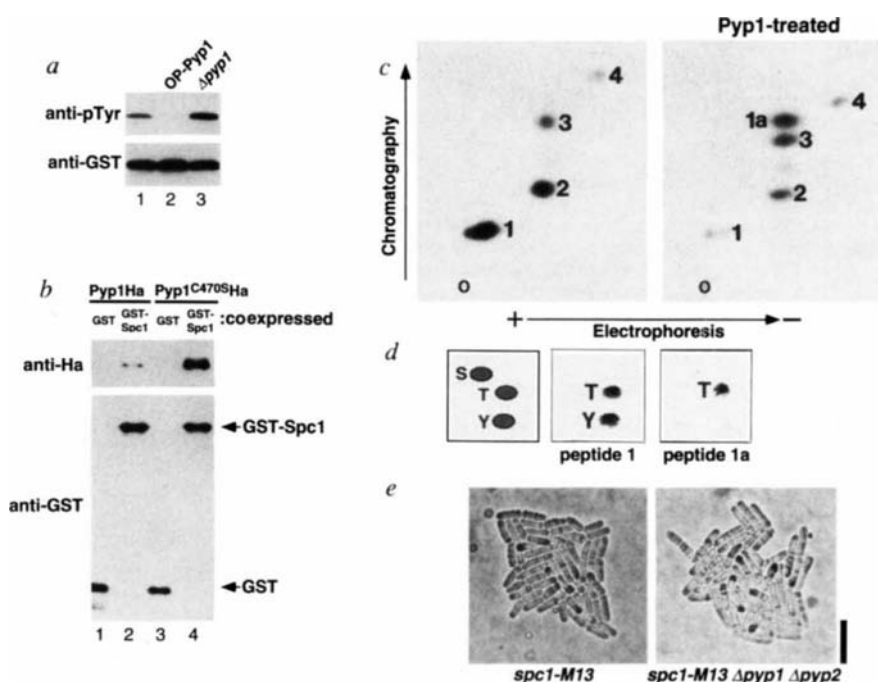
Murine MKP-1 (3CH134/CL100) and *S. cerevisiae* MSG5 were previously implicated as MAPK phosphatases^{4,17}. These phosphatases act as dual-specificity enzymes, dephosphorylating

both threonine and tyrosine residues of MAPKs^{4,18,19}. The catalytic domains of Pyp1 and Pyp2 are more similar to tyrosine-specific phosphatases such as human PTPB (28–31% identical) than to MKP-1 or MSG5 (9–13% identity), raising the issue of whether Pyp1 acts as a mono- or dual-specific phosphatase. GST-Spc1 was phosphorylated with Wis1 and then treated with GST-Pyp1, GST-Pyp1^{C470S} or GST alone. The major tryptic phosphopeptide in the GST-treated sample migrated with the properties predicted for the doubly phosphorylated 165–177 peptide (labelled 1 in left panel, Fig. 4c). As expected, this phosphopeptide contained phosphothreonine and phosphotyrosine (Fig. 4d, middle panel). Treatment with GST-Pyp1^{C470S} had no effect

FIG. 4 a, Pyp1 regulates the tyrosine phosphorylation level of Spc1. GST-Spc1 was expressed from pREP1-GST-Spc1 in wild-type (PR109) transformed with pREP2 (lane 1); PR109 cells transformed with pREP2-Pyp1 (lane 2); and a $\Delta pyp1$ strain (PR253, lane 3). Immunoblotting of total lysates using anti-pTyr and anti-GST antibodies showed that GST-Spc1 tyrosine phosphorylation was abolished in Pyp1-overproducers (lane 2) and increased ~fivefold in $\Delta pyp1$ cells (lane 3). b, Physical association of Spc1 kinase and Pyp1 phosphatase. Ha epitope-tagged Pyp1 (lane 1 and 2) and Pyp1^{C470S} (lanes 3 and 4) were expressed using the *nmf1*⁺ promoter in strains carrying either pREP2-GST (lanes 1 and 3) or pREP2-GST-Spc1 (lanes 2 and 4). Lysates were absorbed with GSH-Sepharose beads. After extensive washes, proteins bound to the beads were analysed by immunoblotting using anti-HA and anti-GST antibodies. Pyp1 and Pyp1^{C470S} bound specifically to GST-Spc1. Interaction between Pyp1^{C470S} and GST-Spc1 was dramatically enhanced. Immunoblotting confirmed that Pyp1 and Pyp1^{C470S} were expressed at equal levels. Coomassie blue staining indicated that GST-Spc1:Pyp1^{C470S} ratio was ~4:1 in lane 4. c, Pyp1 specifically dephosphorylates Tyr-173 of Spc1 *in vitro*. GST-Spc1 was phosphorylated with GST-Wis1 in the presence of [γ -³²P]ATP. The reaction mix was then incubated with GST, GST-Pyp1 or GST-Pyp1^{C470S}. GST-Spc1 treated with GST (left panel) or GST-Pyp1 was isolated by SDS-PAGE and subjected to two-dimensional tryptic phosphopeptide analysis. GST-Spc1 treated with GST-Pyp1^{C470S} appeared identical to the GST-treated sample (data not shown). The major peptide in the left panel, labelled 1, migrated very little from the origin (labelled o) in the electrophoresis dimension. This was expected of doubly phosphorylated 165–177 tryptic peptide, which should have no electrical charge. The major peptide in the right panel, labelled 1a, which appeared following treatment with GST-Pyp1, migrated with the properties expected of the 165–177 tryptic peptide phosphorylated on a single site, having an electrical charge of +1. The identities of the minor phosphopeptide species (2–4) are unknown, they might be derived from partially degraded GST-Wis1 that was autophosphorylated. Phosphopeptide 2 contained phosphotyrosine and appeared to be partially dephosphorylated by GST-Pyp1. d, Two-dimensional phosphoamino-acid analysis of peptides 1 and 1a shown in c. Peptide 1 contains phosphothreonine and phosphotyrosine, whereas peptide 1a contains only phosphothreonine. e, The *spc1-M13* mutation rescues $\Delta pyp1$ $\Delta pyp2$ lethality. Random spore analysis of a cross between strain KS1327 (*h leu1-32 ura4-D18 spc1-M13 pyp1::LEU2*) and KS1328 (*h⁺ leu1-32 ura4-D18 spc1-M13 pyp2::ura4⁺*) produced viable salt-sensitive *Leu⁺ Ura⁺* segregants. The *spc1-M13 pyp1::LEU2 pyp2::ura4⁺* genotypic designation of two *Leu⁺ Ura⁺* segregants was confirmed by outcrossing. The *spc1-M13 $\Delta pyp1$ $\Delta pyp2$* cells divided at 17.7 ± 1.0 μ m. They were indistinguishable from *spc1-M13* single mutant cells.

METHODS. Strains transformed with pREP-derived plasmids were grown for 18 h in EMM2 to induce the *nmf1* expression. To create the *pyp1-C470S* mutant, primers at the 5' (NDE-Y1) and 3' (Y1-NOT) ends

of the *pyp1* ORF were used with C470S/S (5'-AAT ACT ATT GTG CAC TCC TCT GCC GGT GTT GGT-3') and C470S/AS (5'-ACC AAC ACC GGC AGA GGA GTG CAC AAT AGT ATT-3') (the codons corresponding to the substitution from Cys to Ser are in bold; *Apa*LI restriction sites are underlined). PCR amplification was performed with NDE-Y1 and C470S/AS, and another reaction was with C470S/S and Y1-NOT. The products of those reactions were digested by *Nde*I/*Apa*LI and *Apa*LI/*Not*I, respectively, and ligated together into pREP1-Ha6H. For *in vitro* dephosphorylation of GST-Spc1, GST-Spc1 purified on GSH-Sepharose from a $\Delta wis1$ strain (KS1359) was washed and resuspended in 100 μ l of P buffer without 2-mercaptoethanol. 1 μ l of 100 mM FSBA (5-fluorosulphonylbenzoyl-adenosine, in DMSO) was added and incubated for 15 min at 30 °C. This treatment was repeated twice. After washing with P buffer three times, the beads were incubated with a cell lysate of strain KS1384 (a *spc1-M13* strain expressing GST-Wis1) in L buffer to collect GST-Wis1. Beads containing GST-Spc1 and GST-Wis1 were washed extensively in L buffer followed by three washes in P buffer. The phosphorylation reaction was performed in P buffer by adding 100 μ M [γ -³²P]ATP and incubating at room temperature for 60 min. After washing with D buffer (40 mM HEPES pH 7.5, 15 mM NaCl, 2 mM DTT), the reaction was divided into three aliquots. They were mixed with GSH-Sepharose beads containing either GST, GST-Pyp1, or GST-Pyp1^{C470S} proteins purified from a wild-type background. The dephosphorylation reaction was performed in D buffer containing 5 mM glutathione and 0.1 mg ml⁻¹ ovalbumin for 20 min at 30 °C. The ³²P-labelled GST-Spc1 band was cut out from the SDS-PAGE gel after autoradiography. Tryptic peptide mapping and phosphoamino acid analysis has been described²⁸. A pH 1.9 buffer was used for the first dimension electrophoresis, phospho-chromatography buffer was used for the second dimension ascending chromatography.



on the phosphopeptide map (data not shown). Treatment with GST-Pyp1 eliminated phosphopeptide 1 and produced a new phosphopeptide labelled 1a (Fig. 4c, right panel) that contained only phosphothreonine (Fig. 4d). These data strongly indicate that Wis1 phosphorylates Thr 171 and Tyr 173 of Spc1, and that Pyp1 dephosphorylates only Tyr 173.

Double mutant $\Delta pyp1 \Delta pyp2$ spores fail to germinate, indicating that Pyp1 and Pyp2 share an essential function^{2,3}. If this function is to dephosphorylate Spc1, the *spc1-M13* should rescue $\Delta pyp1 \Delta pyp2$. A cross between *spc1-M13* $\Delta pyp1$ and *spc1-M13* $\Delta pyp2$ strains produced viable, salt-sensitive *spc1-M13* $\Delta pyp1 \Delta pyp2$ segregants. The triple mutant was indistinguishable from *spc1-M13* cells (Fig. 4e). These results suggest that Spc1 is regulated by Pyp2 as well as Pyp1.

These findings establish that the *S. pombe* Wis1-Spc1 kinase cascade senses nutrient and osmotic conditions and promotes mitosis. Uncovering the downstream components of the pathway is a goal of current studies. MAP kinases and Cdc2 kinases are proline-directed kinases, thus it is formally possible that they share substrates. However, we favour the possibility that Spc1 regulates transcription factors that are important for promoting mitosis. Our studies demonstrate that MAPKs are regulated by tyrosine-specific phosphatases *in vivo*. This conclusion is consistent with genetic studies that identified *S. cerevisiae* Ptp2, a presumptive tyrosine-specific phosphatase, as a potential regulator of Hog1²⁰. Other studies suggest that mammalian ERK1/ERK2 MAPKs may also be regulated by tyrosine-specific phosphatases²¹. PP2C is a logical candidate for the enzyme that dephosphorylates Thr 171 of Spc1⁵. This idea would be consistent with studies of Hog1 regulation²⁰. However, PP2C and Pyp1 overexpression phenotypes are not identical²², so it is more likely that PP2C regulates another component of the signal transduction pathway.

Note added in proof: Mutations of *spc1* and *wis1* were recently isolated as suppressors of $\Delta pyp1 \Delta pyp2$ (ref. 29). □

Received 5 June; accepted 12 October 1995.

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ACKNOWLEDGEMENTS. We thank M. Shiozaki for technical assistance, C. McGowan for technical advice and comments on the manuscript and B. Edgar and C. Norbury for the *S. pombe* cDNA library. G. Feiser, I. Wilson and S. Reed kindly provided antibodies. K.S. was supported by fellowships from the Human Frontier Science Program and American Cancer Society, California Division. This research was supported by an NIH grant awarded to P.R.

Crystal structure of SIV matrix antigen and implications for virus assembly

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SIMIAN immunodeficiency virus (SIV) is closely related to human immunodeficiency virus (HIV)¹, their matrix antigens (MAs) sharing some 50% sequence identity. MA is a component of Pr55Gag, the sole protein required for assembly of the virion shell². MA targets Pr55 to the plasma membrane³, and facilitates incorporation of the virus envelope protein⁴ and assembly of the Pr55Gag shell⁵. Cleavage of Pr55 by the viral protease produces the mature protein of relative molecular mass 17–18K, which underlies the host-derived membrane and is important in both virus entry⁶ and nuclear localization of the virion core⁷. Here we report the crystal structure of SIV MA. The molecule forms a trimer consistent with oligomerization *in vitro*⁸, the observed virion architecture⁹, and various biological properties of MA.

The crystal structure was determined by multiple isomorphous replacement and cross-averaging (Table 1). Structures at 100 K and 293 K are highly similar (r.m.s. deviation 0.18 Å between Ca atoms). Five amino-terminal residues, which include the site of myristoylation⁵ and 16 carboxy-terminal residues, which include the site of proteolysis, are markedly disordered, consistent with the flexibility necessary for accessibility to post-translational modification enzymes. The structure comprises five α -helices and two 3_{10} helices (H1–H7; Fig. 1a) packing around the central long hydrophobic helix (H6). It broadly resembles the NMR structures of HIV-1 MA^{10,11}, but possesses a previously unobserved 3_{10} helix, H3 (residues 48–52). The region of the three-stranded mixed β -sheet in HIV-1 forms three isolated β -bridges in SIV, residues 20, 28 and 96, reflecting a 6 Å movement of the turn between 22 and 26 and a deviation of 1–2 Å between residues 90 and 98. Beyond residue 110, the C terminus is completely different in both conformation and sequence (1 identity in 9 residues), with residues 110–111 (β 1) and 116–117 (β 2) participating in a β -hairpin. The SIV hairpin is pinned back onto the body of the molecule by Met 118, whose hydrophobic side chain insinuates itself into the bulk of the molecule. Superimposition¹² with a refined NMR structure (S. Matthews, unpublished results) yields 100 equivalences with a deviation of 2.1 Å, twice that expected from the sequence similarity¹³, suggesting inherent flexibility in the molecule. Particular deviations occur at the points of trimer contact (see below) and also in the position of H4. A concentration of large hydrophobic residues is observed around the inner surface of H4; leucines 41, 46, 50, 51 and 64, Cys 57 and Val 82 all lie within a 5 Å radius of Ile 60, producing a rather loose core, sufficiently well packed to exclude water molecules and maintain rigidity in the crystal (indicated by low *B*-factors). This is reminiscent of another viral structural protein, blue-tongue virus VP7 (ref. 14), where the conforma-

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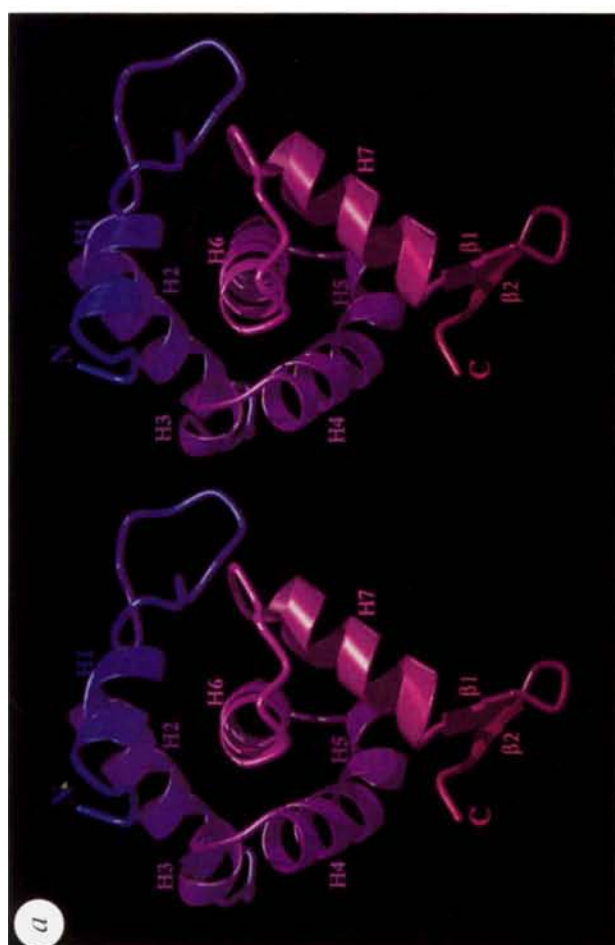
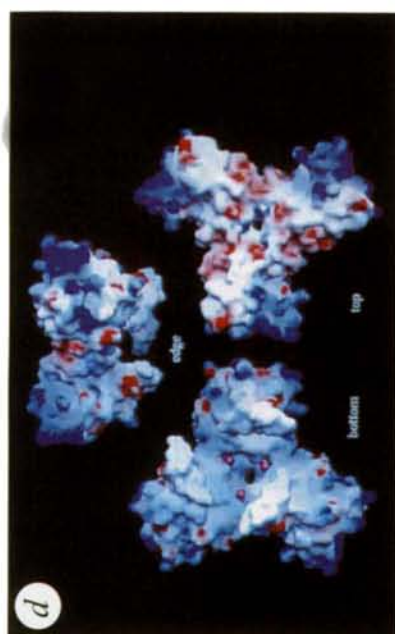
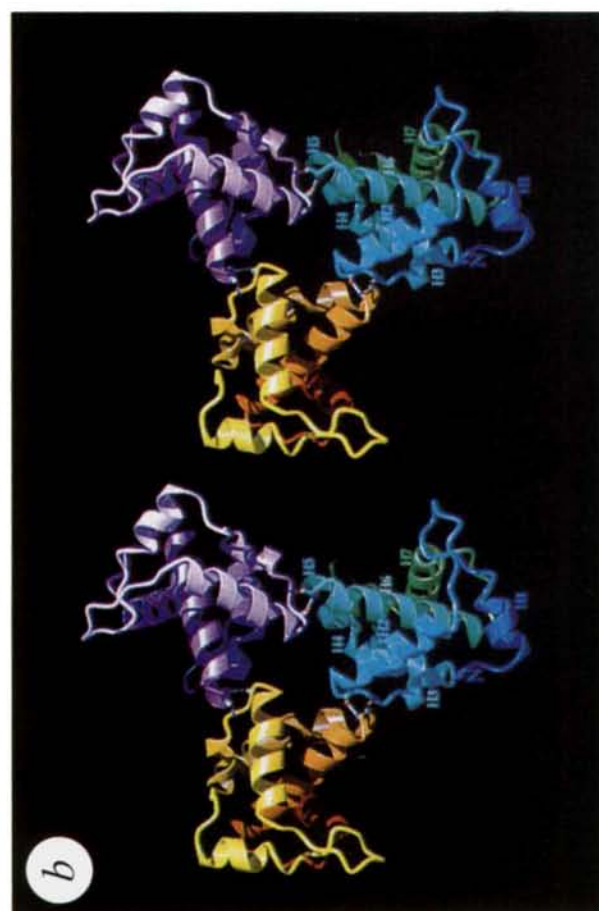
TABLE 1 Structure determination and refinement

Processing statistics									
Data set	Number of crystals	$d_{\max}$ (Å)	Total reflections	Unique reflections	Per cent complete	R_{merge} (%)	MFID (%)	FM	$\langle F \rangle / \langle E \rangle$
100K									
Native	1	2.1	43,654	8,719	97.9	5.9	—	—	—
K ₂ Au(CN) ₄	1	3.5	7,894	1,871	97.4	11.0	23.5	0.58	1.4
K ₂ HgI ₄	1	3.5	6,876	1,515	78.7	10.3	26.8	—	1.0
TAMM	1	3.2	5,617	1,973	78.4	10.1	18.3	—	1.0
Pb(suba.)	1	3.2	8,432	2,477	97.6	7.7	24.1	—	0.2
293K									
Native	4	2.2	21,329	7,686	98.2	7.2	—	—	—
OsO ₄	1	3.5	4,700	1,929	96.6	13.0	21.9	0.28	0.6
EMP	1	4.0	1,650	974	49.5	7.7	24.7	—	0.9
Refinement statistics									
Unit cell (Å)	100 K			293 K					
Resolution range	$a = b = 113.3$, $c = 31.9$			$a = b = 114.9$, $c = 32.3$					
No. reflections: working (test)	30 Å–2.1 Å			30 Å–2.2 Å					
R_{factor} (R_{free})	8,237 (482)			7,686 (—)					
Number of protein atoms	18.0% (24.3%)			16.4% (—)					
Solvent sites	894			894					
R.m.s. deviations from ideality	98 + 2 isopropanols			74 + 1 isopropanol					
Bond lengths	0.010 Å			0.009 Å					
Bond angles	1.4°			1.3°					
$\langle \text{Temperature factor} \rangle$ backbone	27 Å ²			36 Å ²					
Temperature factor deviations	Bond related 3.7 Å ²			Bond related 4.7 Å ²					

$R_{\text{merge}} = \sum_i \sum_h |I_{i,h} - \langle I_h \rangle| / \sum_i \sum_h \langle I_h \rangle$ where h are unique reflection indices, $I_{i,h}$ are intensities of symmetry redundant reflections and $\langle I_h \rangle$ is the mean intensity. MFID is the mean fractional isomorphous difference. FM is the overall figure of merit: the cosine of the mean phase error of the phases. $\langle F \rangle / \langle E \rangle$ is the phasing power: the r.m.s. value of F_h divided by the r.m.s. lack of closure error. $R_{\text{factor}} = \sum_h |F_o|_h - |F_c|_h / \sum_h |F_o|_h$, where h are the unique reflection indices. The expression, purification and crystallization of SIV(mac BK28) matrix antigen (~14K) have been described²⁰. Data were processed using the program DENZO²¹. From the agreement of equivalent reflections the true space group of the crystals was found to be R3, with cell dimensions, in the hexagonal indexing scheme, as shown here, and one molecule in the crystallographic asymmetric unit. **Phase determination.** Attempts to determine the structure by molecular replacement using NMR structures¹¹ failed. Initial phases were obtained by stabilizing crystals in 0.1 M Tris-HCl (pH 8.0), Bis (pH 6.5) or HEPES (pH 7.3) buffer with 25% PEG8000, 15% isopropanol, and adding (at room temperature) various heavy-atom reagents. Many reagents destabilized the crystals in the X-ray beam; however, useful data sets were collected at 20 °C for two derivatives. These data and a native data set were collected in-house using an 18-cm-diameter MARresearch imaging plate, with X-rays supplied by a Rigaku RU-200 and Cu K α radiation selected by reflection from a crystal of graphite. High-resolution room-temperature native data were collected at the Photon Factory, KEK, Japan, station BL-6A2, $\lambda = 0.97$ Å, data collected as 5° (30-s exposure) Weissenberg oscillations on Fuji BAS-III imaging plates scanned off-line using a BA100 scanner. Heavy-atom positions were determined from difference Patterson maps. Heavy-atom refinement was performed using the CCP4 (ref. 22) version of MLPHARE. Solvent flattening (55% solvent content, corresponding to one molecule per asymmetric unit) was performed using GAP (J. Grimes and D.I.S., unpublished) embedded in the CCP4 program suite. During the phase combination procedure, the phases derived from map inversion were downweighted to counteract the inappropriateness of most available weighting schemes. The resulting map at 4 Å was not fully interpretable and further phase information was obtained from cooled crystals. All data collection from cooled crystals used station 7.2 at the DRAL SRS ($\lambda = 1.488$ Å) on an 18-cm-diameter MARresearch imaging plate detector. Some of the water of the crystal liquor was replaced by glycerol, to a final glycerol concentration of 20%. This allowed crystals to be cooled, mounted within loops, to 100 K (Oxford Cryosystems Cryostream device). At this temperature, heavy-atom reagents that had previously rendered the crystal lifetime impossibly short could be examined, and data were collected for several derivatives, together with a low-temperature native data set. The procedures for phase determination were as for the room temperature data. A 4 Å electron density map, obtained by averaging 100 K and 293 K maps after optimal superposition (program GAP), was readily interpreted in conjunction with the NMR structures^{10,11}. **Model building and refinement.** Model building (program FRODO²³) and refinement (program XPLOR²⁴) using Engh and Huber parameters²⁵ was straightforward. This proceeded by building into a map obtained as the average of room temperature and 100 K combined (calculated and MIR) phase maps followed by refinement against the 100 K data set. Rigid-body refinement then placed this structure in the room-temperature cell and limited refinement in this cell followed. These two models then formed the basis for the next round of map calculation. Averaged maps calculated in this way exhibited almost undetectable bias. Refinement weighting schemes were crudely optimized by reference to the R_{free} calculated on 5% of the 100 K data set. Towards the end of the refinement, unaveraged maps were used to rebuild the two structures independently. The final models were optimized by refinement against the complete sets of data (using the weights adopted by reference to R_{free}). Solvent correction used the method implemented in version 3.1 of XPLOR²⁴. For both models, some 95% of the residues lie in the most favoured region of the Ramachandran plot; no residues are in disallowed regions. The protein structure is essentially identical at the two different temperatures (r.m.s. deviation for C α atoms, 0.18 Å), but in some places the electron density is better defined for the lower-temperature structure—this is reflected in the mean B factors for the two structures. There is marked similarity in the water positions (82% of the waters in the room-temperature structure lie within 1 Å of a water molecule in the 100 K structure).

FIG. 1 The structure of SIV MA. a, A stereo view of the monomer (residues 6–119) viewed down the long central helix (H6). Secondary structural elements (as defined in Fig. 2a) are labelled. The molecule is colour-ramped from blue (N terminus) to red (C terminus). b, The crystallographic trimer, viewed from the upper (N-terminal) surface. The monomers are differentiated by being colour-ramped from yellow to red, white to lilac and blue to green, respectively. Secondary structure elements are labelled for one monomer. Trimer contacts between residues Gly 45 and Ser 72 and between Ala 47 and Thr 70 are depicted in ball-and-stick representation with standard atom colouring and hydrogen bonds shown as dotted lines. c, The trimer viewed from the side and oriented such that the N termini are uppermost and the C termini at the bottom; a, b and c were produced using BOBSRIPT, a version (R. Esnouf,

unpublished) of MOLSCRIPT²⁶ and rendered using Raster3D²⁷. d, A GRASP²⁸ representation of the electrostatic properties (using conventional colouring) of the top, bottom and edge views of the trimer showing the amphipathic N-terminal surface and the hydrophobic C-terminal surface. The position of a putative isopropanol alcohol bound in both the 100 K and 293 K structures is indicated by a red sphere. This molecule is observed at both 100 K and 293 K and appears to form a hydrogen bond to the hydroxyl group of Thr 70 and hydrophobic interactions with Leu 67, Leu 46 and the 45–46 peptide backbone. These interactions are consistent with the molecule being isopropyl alcohol (which is present at high concentration) and we have modelled it as such, although the electron density would be equally well explained by other molecules.



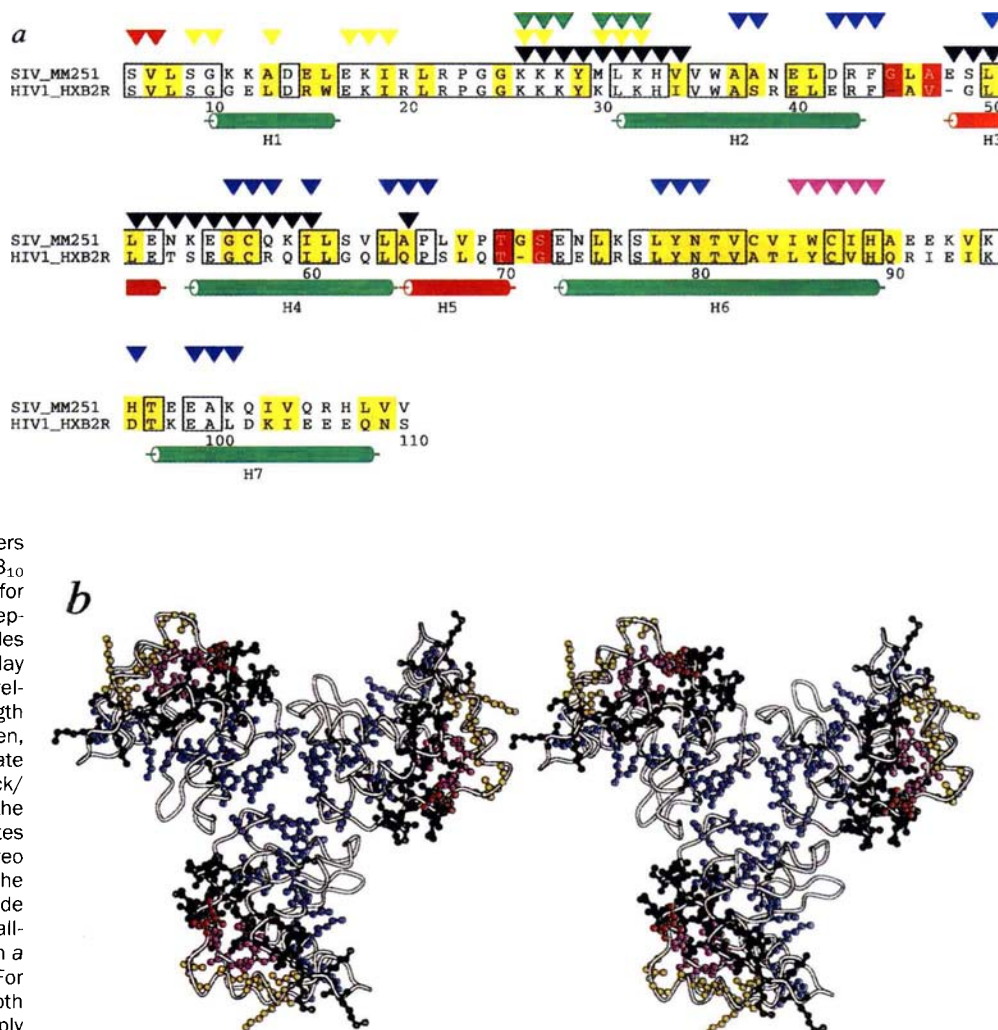
tional plasticity of a greasy protein core might facilitate the promiscuous interactions needed for assembly.

In the SIV MA crystal the molecules are organized around a crystallographic 3-fold axis to produce a trimer (Fig. 1b), which may be a fundamental assembly intermediate of the Gag shell. Oriented such that the N termini approach the upper surface and the C termini trail from the lower surface, the trimer resembles a truncated cone with an upper diameter of some 70 Å, tapering to a lower diameter of 40 Å and spanning a depth of 30 Å (Fig. 1c). This oligomer, oriented with the 3-fold axis radial to the Gag shell, is consistent with many functions of MA. Residues implicated in location, assembly and virion budding⁵ map to the N-terminal portion, forming the upper amphiphilic surface (Fig. 1d). The undersurface of the molecule is hydrophobic and the C termini are well placed to interact with p24 (Fig. 1b), in line with the prediction that the intact Gag protein forms a tapering cylinder¹⁵. Viewed down the axis of the trimer from the assumed external surface (Fig. 1b), the monomers abut cyclically, stabilized by hydrogen bonds between the carbonyl oxygen of Gly 45 and the backbone nitrogen of Ser 72 and between the backbone nitrogen of Ala 47 and the carbonyl oxygen of Thr 70. The use of main-chain groups as the points of trimer contact may ameliorate the structural consequences of the high sequence variation observed in retroviruses (main-chain contacts promoting oligomerization are rather common in viral capsid structures^{14,16}). Additional interactions come from a largely hydrophobic small molecule sandwiched between the three-fold related molecules on the hydrophobic undersurface (Fig. 1d). The surface area

obscured upon trimerization is only 410 Å², a relatively weak multimeric association (in line with the monomeric solution structure), but nonetheless highly specific. Negligible sequence similarity in the C termini of SIV and HIV-1 MA (Fig. 2a) suggests no specific role in assembly consistent with the trimer contacts observed. Mutations that abolish assembly of the Gag shell and MA oligomerization *in vitro*⁸ map to H4 (residues 55–64)⁵ (Fig. 2b). MA trimer interactions are between residues 70 and 72, an acute knuckle at the junctions of H4 and H6, and residues 45 and 47 which form part of a broad bend at the inflexion between H4 and H2 (Fig. 1b). Thus H4 is a spacer, precisely positioning the interacting domains for presentation to the adjacent molecule. Mutations in H4 could uniquely affect both intermolecular contacts at the same time, explaining the effect on assembly. Most amino-acid changes, however, even at the sites of interaction, are silent, as has been shown for mutation at Ser 72 which is assembly-proficient⁵.

In the context of the observed MA trimers, the largely hydrophobic globular body of MA would be separated from the adjacent Gag domain (p24) within the Pr55Gag precursor by H7, suggesting that the structure of mature MA protein described here will be also the structure of MA in the context of Pr55Gag. The architectures of both HIV and SIV are likely to be icosahedral⁹ with the assembly unit a six-membered Gag ring visualized by electron microscopy¹⁵. MA trimers would be consistent with an icosahedral Gag shell in which 6 member Gag rings are composed of three trimers each donating two MA molecules (Fig. 3a, b). Centre-to-centre spacing of Gag rings is

FIG. 2 Structure-function data mapped onto SIV-MA. a, A structure-based sequence alignment¹² of HIV-1_{HXB2} onto SIV_{MM251}. The alignment is based on 104 equivalences with r.m.s. deviation of 2.6 Å between C α atoms. Residues 1–5 and 120–135 are disordered in the SIV crystal structure. There is no structural similarity and only 3 sequence identities beyond residue 110. Structural deviations are also observed in the trimer contact regions. Sequence identities are boxed and the residues constituting the core of the molecule (relatively inaccessible to a water probe) are yellow. Residues losing accessibility on formation of the trimer are coloured red. The helices (as assigned using DSSP²⁹) are denoted by cylinders underneath the alignment, red for 3₁₀ helices and green for α -helices. Data for point mutations, small deletions and peptide inhibitors are indicated by triangles above the alignment: red, block/delay infection⁵; black, block assembly^{5,8}; yellow, block the incorporation of full-length wild-type HIV-1 env glycoproteins⁴; green, block the ability to integrate and replicate in growth-arrested cells⁷; blue, block/delay replication⁵; pink, redirection of the majority of virus particle formation to sites within cytoplasmic vacuoles⁵. b, A stereo view of a trace (MOLSCRIPT²⁶) of the trimer viewed as in Fig. 1b, with the side chains for mutated residues shown in ball-and-stick representation, coloured as in a to denote the effect of the mutation. For clarity, residues implicated in both assembly and another function are simply shown in black.



reported as ~ 66 Å (ref. 15), close to the rhombohedral unit cell dimension of our crystal (68 Å) and a small rearrangement of the crystal lattice generates a well packed planar network (Fig. 3a), consistent with the dimensions and appearance of the electron microscope images of the Gag shell¹⁵. This model has the virtues of simplicity and consistence with the circumstantial evidence presented below, but alternative models cannot be excluded. The CA domain of Pr55Gag exists as a dimer¹⁷. Coupling CA dimerization with trimerization via MA provides a mechanism for higher-order virion assembly that would ensure

selection of full-length Pr55Gag molecules, rather than pre-cleaved products, during assembly. Migration of MA to the plasma membrane requires the region of positively charged residues between Lys 18 and Lys 28 (ref. 3). These residues lie in a loop that is presented at the outer periphery of the trimer upwardly skewed, consistent with interaction with the phospholipid of the plasma membrane. Residues 85–89, mutation of which redirects Gag assembly to cytoplasmic vacuoles⁵, form the main length of H6 and lie directly under the outer charged loop, implicating changes in the hydrophobic core of the molecule in the transmission of conformational change. Mutations in the N-terminal region of MA that prevent envelope incorporation into the assembling particle⁴ lie at the tip of the molecule, in the vicinity of the charged region. The proposed assembly of trimers brings these residues together to form a rim around a saucer-like depression (Fig. 3a) which may just be large enough to hold the cytoplasmic tails of the gp41 oligomer. Ability to accommodate the tail may be the basis of MA–Env interaction, explaining why MA mutations that prevent Env incorporation can be relieved by deletion of the Env cytoplasmic tail⁴.

The features of the molecule we have described offer scope for designed antiviral intervention, perhaps by antagonising the trimer interfaces or masking the envelope binding socket. The hydrophobic cavity occupied by a small molecule also presents a target, somewhat reminiscent of the site of action of some antipicornavirus compounds¹⁸. As structure determination of the adjacent Gag cleavage product CA is ongoing¹⁹ a comprehensive description of Gag assembly may soon be possible.

Note added in proof: It has recently been demonstrated that portions of the *env* protein of several retroviruses, including SIV, form stable trimers (P. S. Kim, personal communication). □

Received 14 September; accepted 30 October 1995.

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ACKNOWLEDGEMENTS. We thank A. Burny for the DNA clone; G. Sutton for help with expression; S. Matthews for access to unrefined and refined coordinates; the staff at the Photon Factory Synchrotron source, Japan (especially N. Sakabe), at the DRAL (particularly T. Greenough) and members of LMB for help with data collection; J. Ren, Y. Jones, R. Esnouf, D. Stammers; K. Harlos for facilitating in-house data collection; R. Bryan and R. Esnouf for computing; and S. Lee for photography. I.J. is grateful to M. Nermut and D. Hockley for discussions relevant to virion assembly. This project was supported by the MRC. Atomic coordinates have been deposited with the protein data bank.

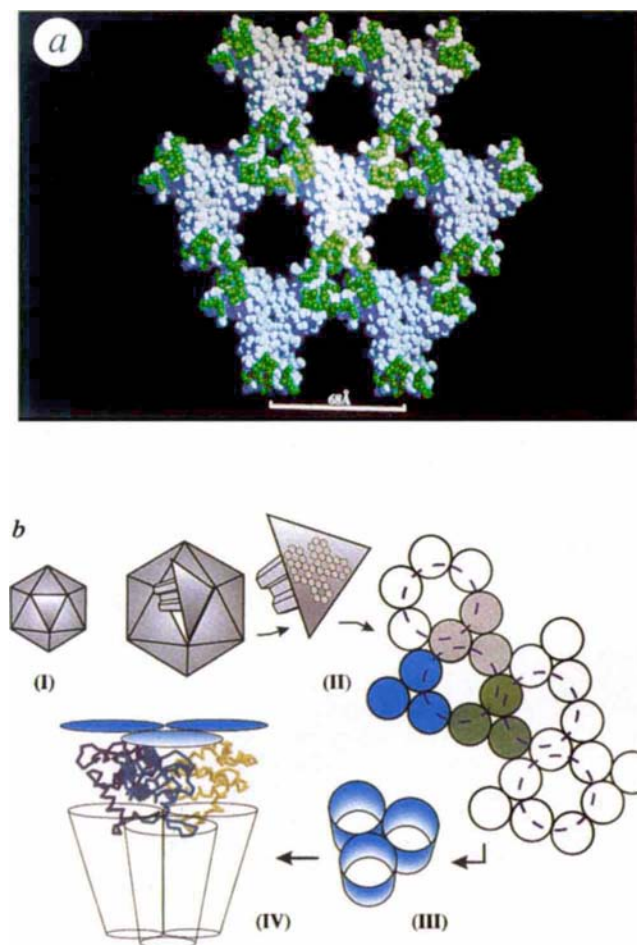


FIG. 3 Higher-order assembly. a, A space-filling representation (grey) generated using MOLSCRIPT²⁶ and Raster3D²⁷ of the network proposed to constitute the MA shell. In this model two principal sets of inter-trimer contacts are observed: between the corner of the molecule involving residues 11 and 24, and also between the corners involving residues 91 and 95–96. The trimer observed crystallographically is shown brighter than the others. Mutations that block the incorporation of full-length wild-type HIV-1 *env* glycoproteins are shown in green, clustering about a surface depression. b, Diagram visualizing the role of the MA trimer in the overall configuration of Gag in the emerging virus. The model represents an *aide memoire* and is not to scale. The architecture of the Gag immature shell is icosahedral (I), with Gag monomers observed to be cylindrical molecules that adopt a hexameric configuration with a centre-to-centre spacing of ~ 66 Å. Trimers (coloured) occur at the junction of each hexamer (broken circle) and provide the inter-molecular contacts necessary for the matrix of Gag to form (II). The trimer has depth and skew (III) which, when viewed from the side and extended for the entire Gag precursor (IV), might generate the curvature of the shell as a natural consequence of the conical nature of this structure.

A 35-Å movement of smooth muscle myosin on ADP release

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MYOSIN II crossbridges interact with F-actin producing power-strokes of around 100 Å (refs 1, 2), during which the products of ATP hydrolysis are released³⁻⁵. This has been postulated to involve an articulation of the myosin head (S1) on actin, or substantial conformational changes in S1 itself⁶⁻⁸. Small movements of the regulatory light chain have been detected (see, for example, refs 9, 10), but most data suggest that the bulk of S1 does not move on actin during crossbridge cycling^{8,11}. Here we present three-dimensional maps of S1-decorated F-actin in the presence and absence of MgADP. The myosin motor domain is similar in both states but there are major orientational differences in the light-chain-binding domain. This domain acts as a rigid level arm^{12,13} pivoting about the end of the motor domain and swinging ~23°, resulting in a ~35-Å step. Small, nucleotide-mediated conformational changes in the motor domain¹⁴⁻¹⁶ may thus be converted by the light-chain domain into large movement steps.

Studies of permeabilized muscle fibres suggest that there are two different, strongly attached actin-myosin-ADP states: one occurring after phosphate dissociation, and a second that is formed following a strain-dependent isomerization^{17,18}. Adding ADP to a fibre induces formation of the latter state, but not the former^{17,19}. In an analogous way, we have created the latter state in a mechanically unstrained system by binding recombinant smooth muscle S1-MgADP to F-actin. The S1 motor and light-chain-binding domains contained residues 1-863 of the myosin heavy chain and both the essential (ELC) and regulatory (RLC) light chains. Cryo-electron microscopy coupled with helical image analysis and averaging^{20,22} was used to obtain three-dimensional (3D) density maps of the nucleotide-free complex (representing the rigor state) and of the MgADP-containing complex (representing an earlier stage in the actomyosin cycle of interaction). The data for the maps of the two structural states are of comparable quality and quantity (Table 1). Both maps contain structural features which, on the basis of previous work^{13,21}, are easily identifiable as the F-actin filament, the motor domain of S1, the ELC and the RLC (Fig. 1).

Comparisons of the two 3D maps reveal that the geometry of interaction of the motor domain with the actin filament is the same in the rigor and ADP-bound states (Figs 1 and 2). This domain has the same angle of attachment and the same bipartite actin binding site^{13,21}, indicating that the contact region does not undergo large changes during the transition from the ADP to the rigor state. It is thus likely that the rearrangements at the large cleft that have been postulated to occur during the crossbridge cycle^{13,16} are complete when phosphate is released.

There are small, but statistically significant, differences in two regions of the motor domain. One region is in front of the actin-myosin interface (Fig. 1, black arrow) and may involve changes in actin, myosin or both. The second difference (Fig. 1, white arrow) lies in the region containing the 25K/50K loop^{12,13} (see Fig. 3), which is involved in modulating nucleotide binding and release²³. The structural difference here may indicate an ordered-disordered transition of the loop. Alternatively, rearrangement

TABLE 1 Summary of data and image analysis results

	Datasets*	Molecules†	Phase residual‡	Resolution of 3D map
MgADP	50	7,303	29 (22-39°)	30 Å
Rigor (no nucleotide)	40	6,082	30° (23-39°)	30 Å

F-actin was prepared from rabbit muscle²⁷. Smooth-muscle S1 was expressed essentially as described in ref. 28. A 'Flag' tag DNA sequence (encoding GDYKDDDDK)²⁹ ligated to the end of cDNA encoding the first 863 amino acids of the chicken gizzard smooth-muscle heavy chain facilitated subsequent protein purification. SF-9 cells were co-infected with recombinant viruses containing coding sequence for the heavy chain and full-length light chains. After 48 h the cells were lysed in an ATP-containing buffer, centrifuged to remove cell debris and a crude S1 fraction obtained by ammonium sulphate precipitation. S1 exhibiting ATP-sensitive actin binding was purified by anti-Flag antibody affinity chromatography. Finally, the S1 was concentrated by vacuum dialysis against low-salt buffer (10 mM imidazole, pH 7.0, 1 mM MgCl₂, 1 mM DTT). This solution—with or without 10 mM MgADP—was used to prepare frozen specimens on holey carbon electron microscope grids^{20,21}. ³¹P NMR spectra of the stock ADP solution indicated no detectable ATP. Grids were examined at ~178 °C in a Philips CM12T electron microscope. Images were recorded 1.5–1.8 µm underfocus using minimal exposure methods (total dose ~10e⁴ Å⁻²). Long, fully decorated filament images were analysed. Decorated filaments of the two states were visually indistinguishable (data not shown) and were similar to those published previously^{20,21}. Helical analysis and averaging was performed as described previously²². Each of the final maps was calculated from 22 layer lines lying within the first zero of the contrast transfer function. The resolution of the 3D maps is ~30 Å in all directions. Statistical difference maps (t maps) were used to assess the significance of the small differences between the two structures^{20,21}. As expected, there were differences of extremely high statistical significance in the light-chain-binding domain of the S1 ($P < 10^{-7}$). In the motor domain there were two regions (see Fig. 1) where the significance of the differences was less dramatic, but still high ($P \approx 10^{-3}$ – 10^{-6}).

* Number of near-side or far-side data sets averaged to give the final data from which the maps were calculated.

† Total number of asymmetric units averaged for each 3D map.

‡ Average amplitude weighted phase residual in degrees obtained when individual near- or far-side data sets were fitted to the final average. The range of values is shown in parentheses. Fitting was performed using the strong peaks of Bessel orders 2, 4, 6, -7, -5, -3, -1, 1, 3 and 5.

of the local secondary structures—similar to that seen in X-ray structures of the motor domain in the presence of nucleotide analogues^{14,16}—could account for the difference. Irrespective of the interpretation, ADP clearly mediates structural changes in the part of the motor domain distant from the cleft and from actin.

In contrast to the overall similarity of the motor domains, the difference in the position of the light-chain-binding domain is dramatic (Figs 1 and 2c). Modelling studies indicate that a good fit between the X-ray structure of chicken skeletal S1 (ref. 12) and our 3D maps is achieved if the light-chain-binding helix is anchored at residue 770 (a conserved glycine) and the entire domain beyond this residue is swung through a ~23° angle (Fig. 2a, b). This results in a ~35-Å movement of the last heavy-chain residue (783) of the X-ray structure in a direction almost parallel to the F-actin filament axis (Fig. 3).

In one respect, this is a surprising result: many models for force generation do not consider the ADP release step as contributing to the powerstroke³⁻⁵, even though there is some evidence that this is the case¹⁷. It has been suggested that the ADP-bound crossbridge may underlie, or at least contribute to, the 'latch' state (a long-lived, high-force crossbridge) in smooth muscle¹⁸. Therefore the nucleotide-induced S1 conformation presented here may represent a 'latch bridge'.

The limited resolution in the maps precludes modelling of precise rearrangements at the pivot point. However, there must be a considerable change in the interactions involving the carboxy-terminal domain of the ELC (specifically helices E and F) and residues in the motor domain (helices containing residues 21–31 and 718–730)^{12,24}. This may explain the altered maximal ATPase activity of myosin isoforms that differ only in ELC C-terminal-domain residues²⁵. Similarly, the idea is also consistent with the observation that some mutations in the β-cardiac myosin heavy chain that are implicated in familial hypertrophic

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cardiomyopathy and impair myosin function, map to this location²⁶.

Several lines of evidence, including efficiency and free-energy considerations, biochemical data, muscle mechanics and power-stroke measurements¹⁻⁵, suggest that the structural changes seen here represent only a part of the working stroke. If the power-stroke is $\sim 100 \text{ \AA}$ ^{1,2}, as much as two-thirds has yet to be seen and must be associated with earlier steps in the cycle. Nonetheless, the data presented here provide compelling evidence for a considerable structural change on ADP release and focus attention on the pivot point where the light-chain-binding helix originates in the motor domain¹². Note that two additional important secondary structure elements converge on this point: the helix

containing the two reactive sulphhydryls (residues 697-709), and a helix (475-507) containing a sequence which is highly conserved throughout the myosin family¹² (Fig. 3). This latter helix originates in the cleft, quite close to the position of the γ -phosphate of ATP¹². Recent X-ray structures of the motor domain in the presence of nucleotide analogues show that both these helices move during ATP hydrolysis^{14,16}. It therefore seems likely that the highly conserved helix and the sulphhydryl-containing helix play major roles in transducing comparatively small structural changes, similar to those occurring at the cleft during hydrolysis, to the pivot point at the end of the motor domain where they are amplified by the light-chain-binding domain into large movements. This hypothesis provides a communication pathway

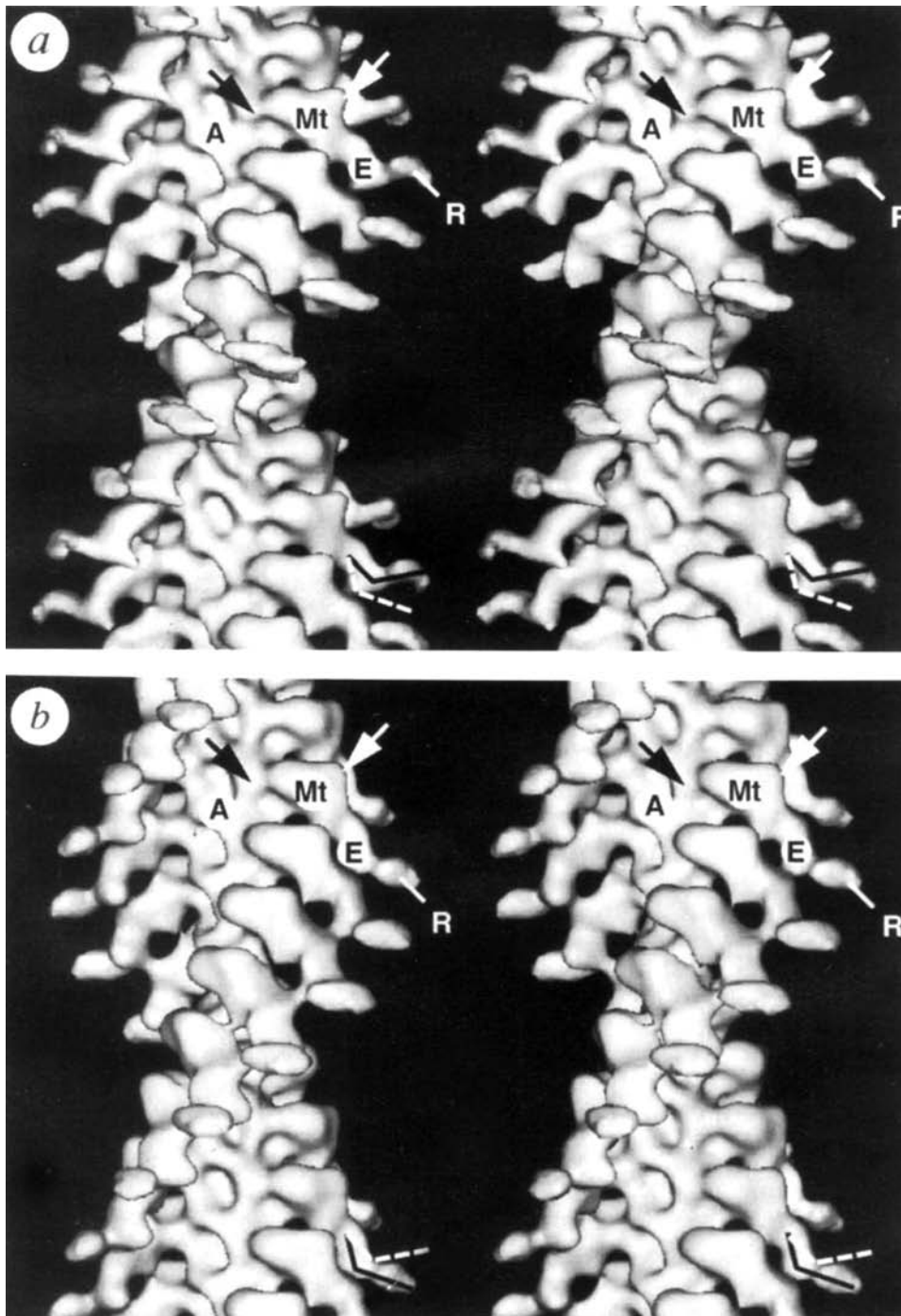


FIG. 1 Stereo pairs of the 3D maps of S1 decorated F-actin *a*, in the presence of MgADP, and *b*, in rigor. Additional control maps of decorated filaments in ~ 50 and ~ 150 mM salt were identical to the rigor maps (data not shown), demonstrating that the differences seen in the presence of MgADP are not due to changes in ionic strength. The assignments for actin (A), the motor domain (Mt), the essential light chain (E) and the regulatory light chain (R) were made on the basis of previous difference mapping and modelling experiments^{13,21}. Black and white arrows identify the parts of the motor which are significantly different in the two maps. In the lower right region of each illustration, solid black lines and dotted white lines emphasize the differences between the two states. This figure was created with SYNU³⁰.

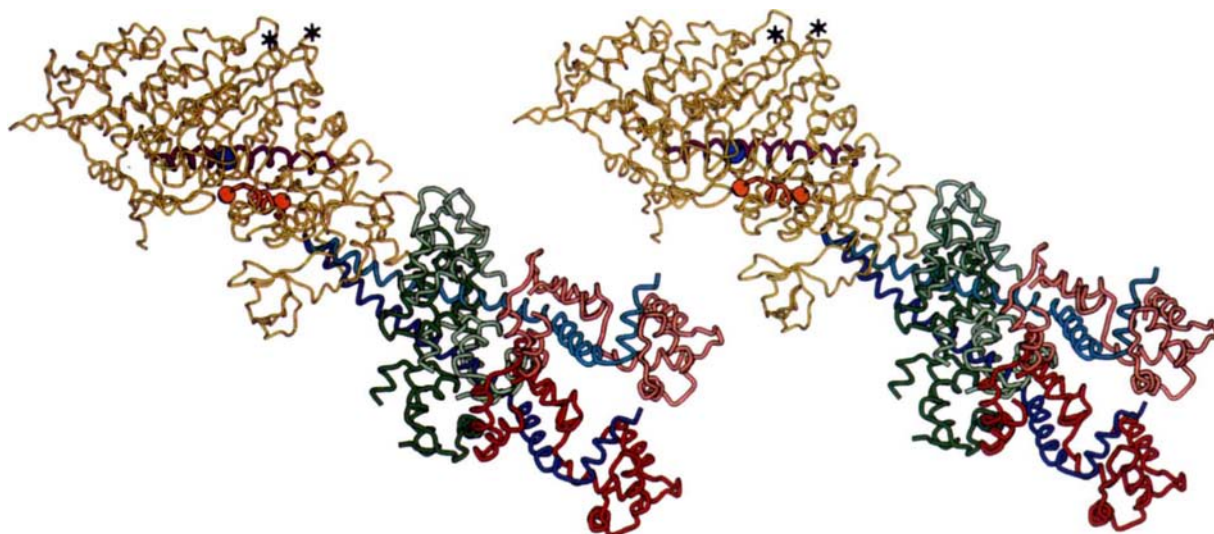


FIG. 2 Stereo pairs illustrating the results of modelling the position of the light-chain-binding domain in the MgADP (a) and rigor (b) states using the X-ray structure of chicken skeletal S1 (ref. 12). The molecular envelope for each state is shown as a red (rigor) or cyan (MgADP) wire frame. The α -carbon backbone of residues 770–843 of the heavy chain (yellow) and of the ELC (white) and the RLC (green) are shown. The rest of the heavy chain has been omitted. In c, the molecular envelopes from the two states have been superimposed to show clearly the overall similarity of the motor domains and the dramatic reorientation of the light-chain-binding domains.

METHODS. Modelling was performed manually using the program O³¹. The clarity of the electron microscopy maps decreases with radius from the filament axis. For this reason the motor domain and the ELC region are well defined, but the RLC is not as well visualized. Optimization of the fit between the ELC parts of the map and the X-ray structure was therefore an important factor in deciding on the best position for the entire light-chain-binding domain. The end result is shown above. Other positions that are not as satisfactory but could in principle be the real locations result in measurements for the angle and the movement which are within $\sim 10\%$ of those cited in the text.

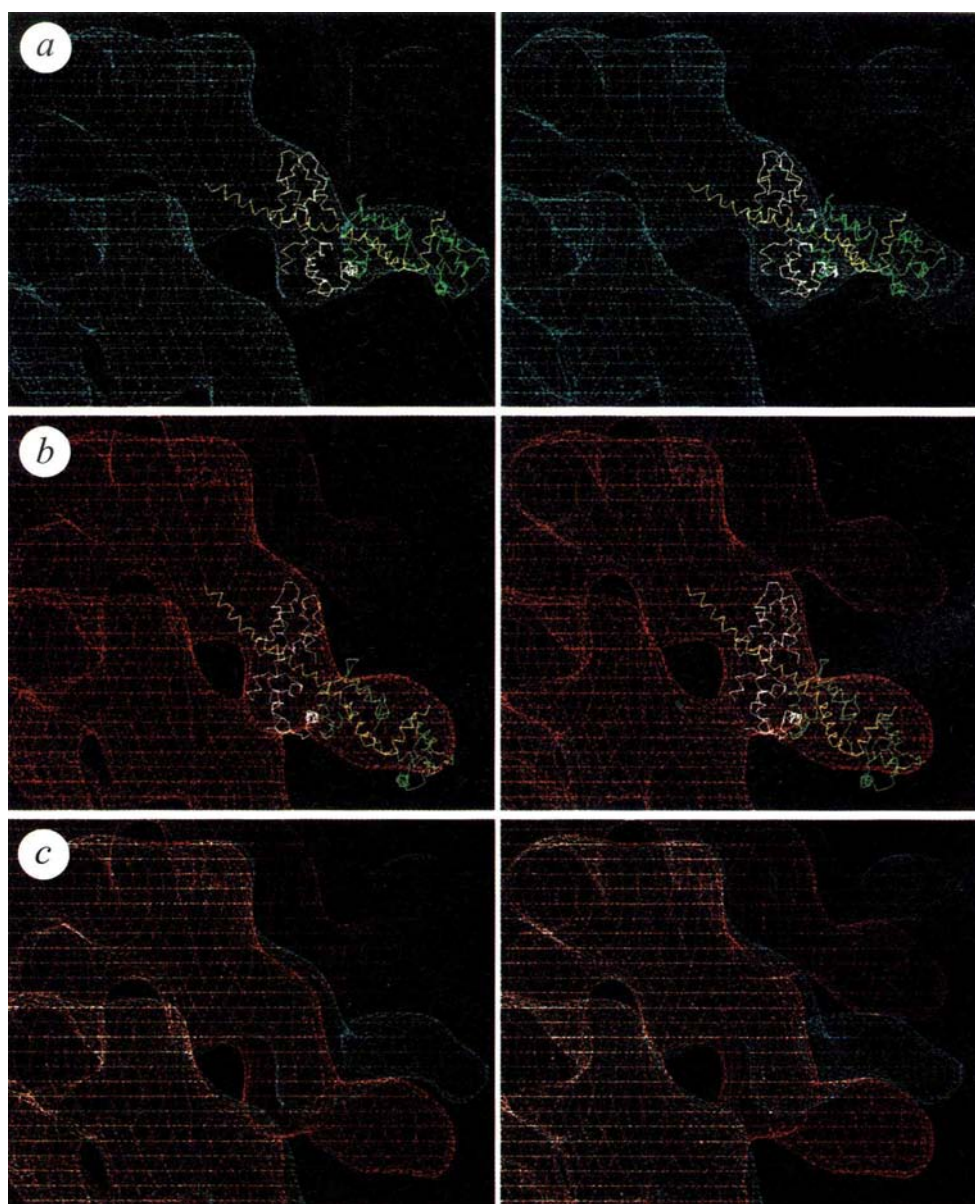


FIG. 3 Stereo pair of the α -carbon backbone of the chicken skeletal myosin S1 molecule¹² with the light-chain-binding domain in the MgADP and rigor positions of smooth-muscle S1. The filament axis runs vertically and the view is from the same angle as Fig. 2. In the light-chain-binding domain, heavy-chain residues 770–843 are blue, the ELC is green and the RLC is red. The darker colours are the rigor position, the lighter colours are the MgADP position. Elements in the motor domain are indicated as follows: the highly conserved helix, residues 475–507, purple; the sulphhydryl-containing helix, residues 697–709, and spheres representing Cys 697 and Cys 707, orange; the approximate location of the β phosphate of the nucleotide, blue sphere; and the 25K/50K loop (asterisks mark the first and last residues; between these residues, the loop is disordered and therefore absent in the crystal structure). Actin monomers to which the head is attached have been omitted for clarity but would lie behind the motor domain. This figure was created with MOLSCRIPT³².

(Fig. 3) linking nucleotide binding and hydrolysis, cleft closure and actin binding, and large-scale movements in a remote region of the molecule. □

Received 16 October; accepted 20 November 1995.

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ACKNOWLEDGEMENTS. A full account of this work and its implications will be published elsewhere. We thank B. Carragher and R. Diaz for help with computer programming and graphics; C. Timmermann for photographic printing and densitometry; L. Marshall for ³¹P NMR spectroscopy; and J. Jontes for helpful discussions. This work was supported by grants from the National Institutes of Health, NIAHS (to R.A.M. & H.L.S.) and the Pew Scholars Program (to R.A.M.). R.A.M. and H.L.S. are established investigators of the American Heart Association.

A 32° tail swing in brush border myosin I on ADP release

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BRUSH border myosin I (BBMI) is a single-headed, unconventional myosin from intestinal microvilli, composed of a heavy chain of relative molecular mass 119,000 (*M_r* 119K) and three calmodulin light chains^{1–3}. Although believed to have a largely structural role, it exhibits the normal actin-activated ATPase and motility properties of a member of the myosin superfamily^{4,5}. Here we present three-dimensional maps of BBMI-decorated actin filaments with and without bound MgADP. While the motor domain remains in a state similar to rigor, the light-chain-binding domain swings through ~32°, resulting in a ~50-Å movement at the end of the region visualized (the second calmodulin light chain). This could correspond to ~72-Å movement of the entire domain. Although qualitatively similar to the movement observed in myosin II⁶, the magnitude of the change is sufficiently different to suggest that structural changes during the actomyosin ATPase cycle differ among myosins, possibly reflecting adaptation for specialized functional demands.

Minimal-dose cryo-electron microscopy⁷ was used to view BBMI-decorated actin filaments either in the absence of nucleotide (rigor state) or in the presence of MgADP (ADP state). Three-dimensional (3D) maps of both states were calculated using helical image analysis^{8,9}, and 19 (rigor) and 28 (ADP) data sets were averaged for the final maps. The final averages are of comparable quality. The region occupied by F-actin and the

BBMI motor domain seems to be indistinguishable in the two maps (Figs 1 and 2). In contrast, the light-chain-binding domain is dramatically different. The observed change is consistent with the idea that this domain swings as a rigid structural unit about a point in the distal portion of the motor domain^{10–13}. This movement represents a ~32° change in angle about the pivot point, which results in a ~50-Å axial translation of the part of the BBMI structure farthest from the filament axis (Fig. 1). Comparisons with maps of F-actin decorated with chicken skeletal S1 (refs 9, 12) suggest that only two of the three calmodulin light chains are visualized in our maps (Fig. 1). The third calmodulin and an additional carboxy-terminal domain are not seen, perhaps owing to limitations in the methodology or data analysis; for example, limited resolution, disorder at high radius, or radiation damage. However, assuming that the light-chain-binding domain is rigid to at least the end of the third calmodulin, we estimate that the BBMI molecule could move ~72 Å as a result of ADP release.

The observed structural change is similar in character to that of smooth-muscle myosin⁶ (SMM): F-actin and the motor domain appear unchanged, and there is a large reorientation of the light-chain-binding region. Although these observations imply conservation of a fundamental underlying mechanism, there are substantial differences in the behaviour of the two myosins. Most importantly, the change in angle of the BBMI light-chain-binding domain is ~32°, representing a swing ~40% larger than is seen in SMM⁶. As well as this quantitative difference, there is an additional variation: the BBMI light-chain-binding domain seems to move azimuthally and rotate clockwise by ~10° about its long axis during nucleotide release. Clearly, the structural consequences of a single biochemical step, in this case ADP release, can differ substantially between myosins, that is, individual structural transitions may not only go faster or slower, but also may vary in magnitude. This observation, if applicable to the rest of the ATPase cycle, could provide a tremendous source for variations in efficiency, velocity and force from which myosins could be tailored for diverse cellular duties.

Our results, and those of Whittaker *et al.*⁶, indicate an ADP-induced structural change in actomyosin. Because neither ATP

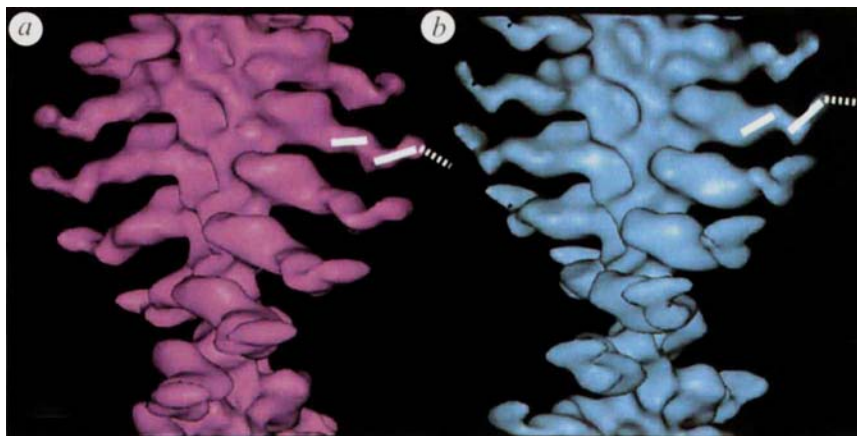
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hydrolysis nor phosphate release is involved, the observed movement must be fuelled by the affinity of myosin for the ADP molecule. Furthermore, the results demonstrate that at least part of the myosin powerstroke is a transition between so-called strongly attached states^{14–18}. This makes sense because myosin could only produce high force if it were already attached firmly to the actin filament¹⁹. It would then be logical to suppose that any earlier steps generating high force would also have to be transitions between strongly attached states. The 'weak-to-strong'^{16–18} transition must then be a two-step process: formation of a firm attachment of myosin to actin, followed by a step generating high force.

In addition to its structural role in the microvillus, BBMI has also been suggested to participate in moving vesicles through the apical cytoplasm of enterocytes^{20,21}. This environment contrasts sharply with that of SMM, which exists in the organized and highly cooperative structure of the myofilament assembly. Although the action of a single SMM molecule does not have a great impact on a macroscopic contraction, the relative contribution of a single motor becomes critical in the case of BBMI, where only a small number of myosins are used in transporting a vesicle along an F-actin filament. Adaptation of BBMI to its environment would be critical for the efficient performance of this duty. The specialized functional attributes necessary for this

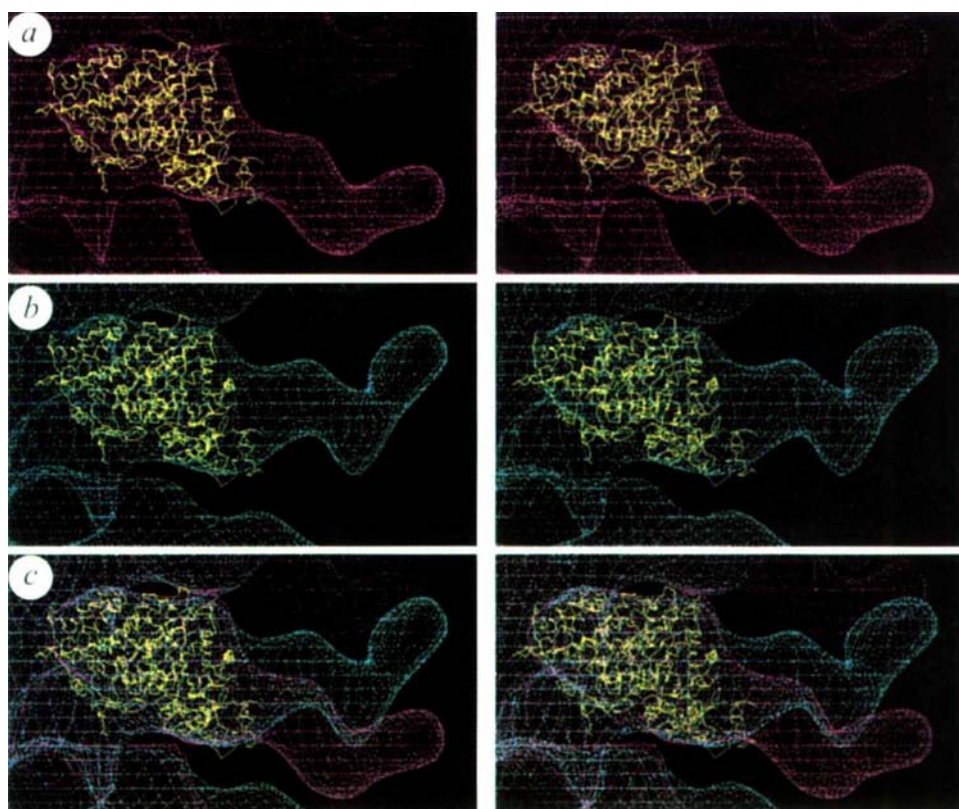
FIG. 1 Structure of BBMI-decorated actin filaments in the absence and presence of MgADP. *a*, The structure shown in purple represents F-actin decorated with BBMI in the rigor state. The light-chain-binding domain can be seen as a thin, irregular tail extending outwards from the motor domain. Solid white bars represent the approximate locations of the two calmodulin light chains seen in the maps. The third calmodulin was not seen, but its estimated location is indicated by a broken white bar. *b*, The F-actin–BBMI–MgADP structure, shown in blue, reveals a remarkable reorientation of the light-chain-binding domain. In comparison to the rigor structure, the visual effect is to make the filament almost appear inverted. As with the rigor structure, there is no density attributable to the third calmodulin light chain.

METHODS. Actin was prepared from rabbit skeletal muscle as described previously²², and BBMI was purified by a modification of a previous method⁴ (J. D. Jontes *et al.*, manuscript in preparation). To prepare specimens for both the ADP and rigor structure determinations, a buffer of low ionic strength was used (10 mM imidazole, pH 7.0, 1 mM DTT, 1 mM MgCl₂, 1 mM EGTA, with or without 10 mM MgADP). Images of frozen-hydrated filaments were recorded in a Philips CM12T electron microscope at a nominal magnification of $\times 35,000$ and a defocus of



$\sim 1.5 \mu\text{m}$. Filament selection, image analysis and averaging were performed as described previously⁶. The rigor and ADP averages were composed of 19 (2,146 molecules) and 28 (2,473) near- or far-side data sets, respectively, giving final amplitude weighted-mean phase residuals of 32 ± 3 (s.e.m.) and 32 ± 4 . The 3D maps shown were rendered using the program SYNU²³.

FIG. 2 Comparison of the BBMI molecule in the ADP and rigor states. *a*, Stereo pair showing the molecular envelope of a single BBMI molecule in the rigor state (magenta). The α -carbon backbone of residues 81–770 of the chicken skeletal myosin heavy chain²⁴—representing the BBMI motor domain—is shown in yellow. The first 80 amino acids were removed from the chicken skeletal S1 heavy chain as there is no homologous region in BBMI²⁵. *b*, Stereo pair of BBMI in the ADP state (cyan), again enclosing the α -carbon backbone of the motor domain (yellow). The backbone location is the same in *a* and *b*, and fits the two molecular envelopes equally well. *c*, Stereo pair in which the rigor and ADP-bound states have been superimposed. This comparison demonstrates that, whereas the light-chain-binding domain pivots as a rigid body, the motor domain does not exhibit large ADP-induced movements. In support of this observation, a statistical difference map (*t*-map) revealed that the only statistically significant differences ($P < 0.001$) between the two density maps lay in the light-chain-binding domain. The figure was created with the modelling program O²⁶.



task might include an increase in the time attached to actin during the ATPase cycle, and an efficient use of the time spent attached. It seems likely that BBMI may have evolved a larger swing and a longer tail to achieve these goals. These ideas underscore the most important implication of the results presented here: the generation of myosins for functional niches is the result of variation in the structural consequences, as well as the rates, of individual biochemical steps in the ATPase cycle. □

Received 16 October; accepted 20 November 1995.

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ACKNOWLEDGEMENTS. We thank M. Whittaker for computational assistance and discussions, R. Diaz for help with creating figures, and H.L. Sweeney for comments on the manuscript. This work was supported by grants from the National Institutes of Health (NIAMS) and the Pew Scholars Program (R.A.M.). R.A.M. is an established investigator of the American Heart Association. J.D.J. is a Howard Hughes Medical Institute predoctoral fellow in the Biological Sciences.

ERRATUM

Multiple essential functions of neuregulin in development

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Nature **378**, 386–390 (1995)

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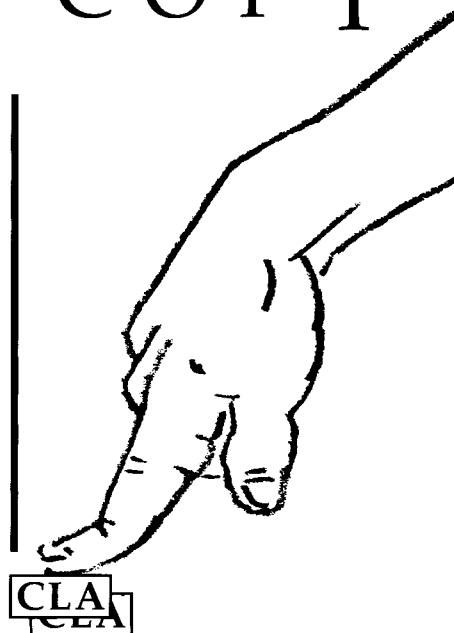
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Scientific experts: more attention needed

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A survey of members of the legal profession involved in cases requiring the use of scientific experts reveals strong support for court-appointed experts — but little evidence of direct experience to back up this feeling.

In English law, the general rule covering the appearance of witnesses in court cases is that their testimony must be restricted to facts, and that tribunals should not be subject to their personal views and opinions.

There are, however, many occasions on which the tribunal lacks the ability to form an opinion on its own on the evidence presented to it. Judges are not polymaths, and where the issue under consideration is one of science (or art), assistance from an expert may be required. As a result, English courts have recognized the need for special witnesses who are able to provide evidence on both fact and opinion.

Yet expert witnesses and their reports have been the subject of criticism by the judiciary for more than 100 years¹, often because judges have to choose between the testimony of two opposing experts, and may feel that expert evidence is partisan. As a result, some judges began to resort to a referee to reach a decision.

Systems of court appointed experts subsequently developed, under which matters of opinion and fact are referred to a single expert appointed by the courts. This has removed the need for both parties to appoint their own expert, and can obviate the often wasteful system of adversarial examination and cross-examination.

Since 1932, Order 40 of the Rules of The Supreme Court² has allowed a judge to appoint a court expert when requested by the parties involved in litigation. In practice, however, this rarely happens. A review of civil litigation is currently being carried out by the government, and various aspects of the use of experts have been addressed in an interim report, prepared under the chairmanship of Lord Woolf³.

The Woolf Report proposes, for example, that the calling of expert evidence should be subject to the complete control of the court, with the implication that in most cases, this will mean the court refusing to allow any oral evidence from experts appointed by the litigating parties.

The report also proposes that courts should have the power to appoint when appropriate a single expert to report or give evidence to the court — if necessary without the agreement of the parties — and to appoint assessors of the facts presented. Experts would also be given clear

guidance that their first responsibility is to the court, and not to their client.

We have carried out research into the area of witness's opinions and the use of court experts. A questionnaire was sent out asking for opinions on the use of experts, and for data on the use of Order 40, among judges sitting as Official Referees — High Court judges specializing in technical disputes — as well as members of the professional associations covering the barristers and solicitors who practise in the courts over which such judges preside.

Replies to the questionnaire revealed that Order 40 is rarely considered, and even more rarely used. Among those respondents with experience of using Order 40, satisfaction with the use of court appointed experts was low and little more than satisfactory, mainly because they tended to be used in parallel to the existing system of parties appointed by the experts.

Furthermore, 85 per cent of respondents indicated that views of expert witnesses often differ, while members of the judiciary were unanimous that differences of opinion between parties' expert witnesses were a significant factor in extending the length of trials; 85 per cent of lawyers agreed.

The questionnaire also sought the opinion of respondents on changing the rules of the Supreme Court so as to increase the use of the court expert by leaving the appointments of such experts to judicial discretion. Almost two-thirds (65 per cent) of the judiciary were in favour of such a move, while three quarters of professionals who had experience of order 40 were also in favour.

The debate on the effectiveness of expert witnesses appointed by the opposing parties in a court case has raged periodically in the United States, most recently in the late 1980s (see ref. 4). A US procedure similar to Order 40 exists, namely Federal Rule of Evidence 706⁵.

The rule was established because of concern over issues such as the practice of 'shopping' for experts — in other words seeking an expert opinion which strongly supports the case of the person paying for the expert — the venality of some of those selected, and the reluctance of many others, particularly those with high reputations, to involve themselves in litigation (see ref. 4).

Nevertheless, a study by the Federal Judicial Center in Washington, D.C., found that only 20 per cent of federal district court judges had ever used their power to

appoint court expert under this rule, and half of those had only done so once⁶.

One area identified by our research in Britain, and reinforced by the other studies published in the US literature, is concern over the qualifications and degree of expertise offered and exercised by experts. Pamela Johnston (see ref. 4), for example, of the University of California, Davis, puts forward powerful arguments for the use of experts as individuals who use their education, autonomy, experience and intuitive understanding, not as assistants to the fact-finder.

The wide degrees of competence found among experts degrades the whole legal system. What is needed is experts who are known to have the necessary expertise, and possess the type of intuition needed to solve problems.

Such intuition is impossible to define formally, although it may be demonstrated by an ability to understand new situations which go beyond current knowledge where, for example, state-of-the-art science or technology is being considered. Scientists may see an opportunity here to use their systematic and formulated knowledge in offering a quality of opinion which may allow justice to be done.

The Woolf Report was set up to review the current rules and procedures of the civil courts in England and Wales with the stated aims of improving access to justice and reducing costs.

Woolf's proposal for court appointed experts to address the problems associated with expert evidence deal with the system and not the experts. We found no evidence that a system of court appointed experts would work, either in this country or elsewhere. There has been very limited use of the current provisions for court-appointed experts. Many in our sample focused on the variety of standards amongst experts. This interface between science and the law requires more attention. □

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ACKNOWLEDGMENTS. This work was funded by The Engineering and Physical Sciences Research Council in collaboration with Masons Solicitors and Privy Council Agents. We thank E. Davies, partner at Masons, for steering the research.